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THE USE OF A TETRAZOLIUM SALT REDUCTION TEST TO STUDY  
METABOLIC DAMAGE OF ESCHERICHIA COLI CAUSED BY EXPOSURE  
TO SOME CHLORINE BEARING DISINFECTANTS

by



GUENTER W. RIEDEL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES  
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The undersigned certify that they have read, and  
recommend to the Faculty of Graduate Studies for acceptance,  
a thesis entitled:

THE USE OF A TETRAZOLIUM SALT REDUCTION TEST TO STUDY  
METABOLIC DAMAGE OF ESCHERICHIA COLI CAUSED BY EXPOSURE  
TO SOME CHLORINE BEARING DISINFECTANTS

submitted by Guenter W. Riedel in partial fulfilment of the  
requirements for the degree of Doctor of Philosophy.



## ABSTRACT

The reduction of 2, 3, 5 - triphenyl-tetrazolium chloride (TTC) by dehydrogenases of Escherichia coli was investigated. The results allowed the development of a simple vital staining test, using 200 ppm TTC for investigating some of the problems involved in chlorine resistance and death of E. coli by chlorine. Concentration of TTC much in excess of 200 ppm affected the test by interfering with the formation of formazan. It could be shown that at least three dehydrogenases (glucose, malic and lactic) are involved and the results suggested that glucose and malate utilization are more susceptible to chlorine inactivation than is lactate utilization. This finding is considered important in the interpretation of time survivor data from disinfection experiments.

A chemostat was used to grow E. coli for exposure to chlorine and it could be shown that reproducibility of disinfectant experiments depended on the care with which the chemostat was operated viz., frequent monitoring and manual adjustment of the flow rate of medium and air and adequate mixing to prevent stratification of medium components. The results emphasized that a condition of steady state in a chemostat should not be judged solely on logarithmic plots of colony counts but also on metabolic activity of the culture.

It was further shown that glucose, which does not appear to react with chlorine, protected bacteria from inactivation by chlorine.





This investigation shows that some organic materials, e.g. glucose may not simply act in a competitive manner by reacting with chlorine, but may actually protect E. coli against the lethal effect of chlorine at the enzyme macromolecular level.



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It is a pleasure to acknowledge the assistance and support of my wife.



"At times it may appear that the reviewer has forgotten that the primary aim of employing radiation in the study of phage is to elucidate the normal state of affairs. However, almost all experiments involving the irradiation of phage have raised far more questions than they have answered. This has resulted in the situation that there now exists, a 'radiobiology of bacteriophage', a collection of observations and hypotheses arising from irradiation experiments, leading no one knows where, but selfishly demanding an explanation."

F.W. Stahl

The Viruses (1959)



## TABLE OF CONTENTS

|                                                                                                                        |    |
|------------------------------------------------------------------------------------------------------------------------|----|
| INTRODUCTION                                                                                                           | 1  |
| DISINFECTION - GENERAL .....                                                                                           | 1  |
| REVIEW OF SELECTED LITERATURE                                                                                          | 11 |
| TETRAZOLIUM SALTS .....                                                                                                | 11 |
| The Chemistry of Tetrazolium Salts .....                                                                               | 11 |
| Biological Applications of Tetrazolium Salts .....                                                                     | 14 |
| Enzymic Reduction of Tetrazolium Salts .....                                                                           | 15 |
| Alkaline Tetrazolium Salt as a Method of Illustrating<br>Protein-Bound Sulfhydryl and Disulfide Groups .....           | 18 |
| The Bactericidal or Bacteriostatic Properties of<br>Tetrazolium Salts and Other Quaternary Ammonium<br>Compounds ..... | 19 |
| The Use of TTC as an Indicator of Bacterial Damage<br>by Chemical Agents .....                                         | 24 |
| DYNAMICS OF DISINFECTION .....                                                                                         | 27 |
| CRITERIA OF DEATH OF MICRO-ORGANISMS .....                                                                             | 34 |
| CHLORINATION .....                                                                                                     | 37 |
| Active Compounds of Chlorine .....                                                                                     | 37 |
| The Effect of Various Chemical Substances of Chlorine ....                                                             | 41 |
| The Effect of Temperature .....                                                                                        | 44 |
| The Effect of Concentration .....                                                                                      | 45 |
| The Effect of pH and the Mechanisms of Disinfections ....                                                              | 46 |
| CONTINUOUS CULTURE .....                                                                                               | 50 |
| Design of Apparatus and Theory of Operation .....                                                                      | 51 |





|                                                                              |    |
|------------------------------------------------------------------------------|----|
| MATERIALS AND METHODS                                                        | 54 |
| PREPARATION AND COMPOSITION OF MEDIA, SUBSTRATES AND OTHER SOLUTIONS         | 54 |
| Bacteriological Media                                                        | 54 |
| Minimal broth                                                                | 54 |
| Complete media                                                               | 55 |
| Solid media                                                                  | 55 |
| Buffered diluent                                                             | 56 |
| Substrates and Solutions for the Tetrazolium Tests                           | 56 |
| Stock Solutions of Chlorine and Chloramine-T                                 | 59 |
| Growth and Maintenance of Bacteria                                           | 59 |
| Maintenance of stock cultures                                                | 59 |
| Preparation of inocula and batch culture                                     | 60 |
| Continuous Culture of <u>E. coli</u>                                         | 60 |
| Description of apparatus used                                                | 60 |
| Sterilization of the culture assembly                                        | 64 |
| Preparation of medium supply for the chemostat                               | 64 |
| Inoculation of the chemostat                                                 | 66 |
| Testing operation of the chemostat                                           | 67 |
| The Preparation of Washed Bacterial Suspensions for Disinfectant Experiments | 67 |
| Washing of Bacteria                                                          | 68 |
| Adjustment of Bacterial Concentration (optical density)                      | 69 |
| Determination of Numbers of Viable Bacteria (spot-plate technique)           | 70 |
| DISINFECTION EXPERIMENTS                                                     | 71 |
| Cleaning of Glassware                                                        | 71 |



|                                                                                                                                                         |        |
|---------------------------------------------------------------------------------------------------------------------------------------------------------|--------|
| Disinfection .....                                                                                                                                      | 72     |
| Tests Using Tetrazolium Salts for Staining Bacteria .....                                                                                               | 73     |
| Preparation of substrate-solution mixtures .....                                                                                                        | 73     |
| The effect of tetrazolium salt on formazan formation by <u>E. coli</u> ..                                                                               | 74     |
| Tetrazolium test for monitoring <u>E. coli</u> growing in the chemostat .                                                                               | 75     |
| Illustration of metabolic damage by chlorine .....                                                                                                      | 75     |
| Conclusion to Materials and Methods .....                                                                                                               | 76     |
| <br>RESULTS AND DISCUSSION .....                                                                                                                        | <br>77 |
| PRESENTATION OF RESULTS .....                                                                                                                           | 77     |
| VITAL STAINING WITH TETRAZOLIUM SALT .....                                                                                                              | 77     |
| Estimation of Viable Bacteria by Extracting Formazan .....                                                                                              | 79     |
| The Effect of Culture History and TTC Concentration<br>on Vital Staining of <u>E. coli</u> .....                                                        | 83     |
| Summary of Vital Staining of <u>E. coli</u> with Tetrazolium<br>Salt .....                                                                              | 96     |
| Maintenance of <u>E. coli</u> in the chemostat .....                                                                                                    | 101    |
| THE INVOLVEMENT OF VARIOUS DEHYDROGENASES IN DEATH OF BACTERIA<br>BY CHLORINE AS ILLUSTRATED BY TETRAZOLIUM SALT REDUCTION .....                        | 108    |
| The Use of Complex and Specific Substrates to<br>Illustrate Metabolic Damage by Chlorine .....                                                          | 108    |
| The use of complex substrates and glucose to illustrate enzymic<br>damage by chlorine .....                                                             | 110    |
| The use of specific substrates (glucose, sodium malate and sodium<br>lactate) to illustrate dehydrogenase damage of <u>E. coli</u> by<br>chlorine ..... | 118    |
| The effect of chloramine on <u>E. coli</u> grown in the chemostat .....                                                                                 | 123    |
| Evidence of substrate protection as studied by pre-incubation of<br>washed <u>E. coli</u> with glucose prior to exposure to chlorine .....              | 127    |





|                                                                                                                                                      |        |
|------------------------------------------------------------------------------------------------------------------------------------------------------|--------|
| Disinfection .....                                                                                                                                   | 72     |
| Tests Using Tetrazolium Salts for Staining Bacteria .....                                                                                            | 73     |
| Preparation of substrate-solution mixtures .....                                                                                                     | 73     |
| The effect of tetrazolium salt on formazan formation by <u>E. coli</u> ..                                                                            | 74     |
| Tetrazolium test for monitoring <u>E. coli</u> growing in the chemostat .                                                                            | 75     |
| Illustration of metabolic damage by chlorine .....                                                                                                   | 75     |
| Conclusion to Materials and Methods .....                                                                                                            | 76     |
| <br>RESULTS AND DISCUSSION .....                                                                                                                     | <br>77 |
| PRESENTATION OF RESULTS .....                                                                                                                        | 77     |
| VITAL STAINING WITH TETRAZOLIUM SALT .....                                                                                                           | 77     |
| Estimation of Viable Bacteria by Extracting Formazan .....                                                                                           | 79     |
| The Effect of Culture History and TTC Concentration<br>on Vital Staining of <u>E. coli</u> .....                                                     | 83     |
| Summary of Vital Staining of <u>E. coli</u> with Tetrazolium<br>Salt .....                                                                           | 96     |
| Maintenance of <u>E. coli</u> in the chemostat .....                                                                                                 | 101    |
| THE INVOLVEMENT OF VARIOUS DEHYDROGENASES IN DEATH OF BACTERIA<br>BY CHLORINE AS ILLUSTRATED BY TETRAZOLIUM SALT REDUCTION .....                     | 108    |
| The Use of Complex and Specific Substrates to<br>Illustrate Metabolic Damage by Chlorine .....                                                       | 108    |
| The use of complex substrates and glucose to illustrate enzymic<br>damage by chlorine .....                                                          | 110    |
| The use of specific substrates (glucose, malic acid and sodium<br>lactate) to illustrate dehydrogenase damage of <u>E. coli</u> by<br>chlorine ..... | 118    |
| The effect of chloramine on <u>E. coli</u> grown in the chemostat .....                                                                              | 123    |
| Evidence of substrate protection as studied by pre-incubation of<br>washed <u>E. coli</u> with glucose prior to exposure to chlorine .....           | 127    |



|                                                                                                                                                              |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Evidence that variation in chlorine-resistance of <u>E. coli</u><br>can be reduced when conditions in growing the bacteria are<br>carefully controlled ..... | 127 |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|

|                    |     |
|--------------------|-----|
| GENERAL DISCUSSION | 132 |
|--------------------|-----|

|            |     |
|------------|-----|
| REFERENCES | 136 |
|------------|-----|

|          |    |
|----------|----|
| APPENDIX | A1 |
|----------|----|





## LIST OF TABLES

|           |                                                                                                                                                                                                   |     |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Table 1.  | The relationship between formazan production and bacterial concentration as illustrated by comparing turbidity of bacterial suspensions and optical density of solvent extracts of formazan ..... | A1  |
| Table 2.  | The effect of TTC concentration on vital staining of <u>E. coli</u> with different culture histories .....                                                                                        | A2  |
| Table 3.  | The effect of TTC concentration on vital staining of <u>E. coli</u> maintained in a chemostat in minimal broth .....                                                                              | A3  |
| Table 4.  | The effect of TTC concentration on vital staining of <u>E. coli</u> grown in complete broth in batch culture .....                                                                                | A4  |
| Table 5.  | The effect of TTC concentration and incubation time on vital staining of <u>E. coli</u> grown in batch culture in complete broth .....                                                            | A5  |
| Table 6.  | The effect of staining <u>E. coli</u> grown in complete broth in batch culture for 20 h at 32°C in the presence of dead bacteria .....                                                            | A6  |
| Table 7.  | The effect of bacterial and TTC concentration and incubation time on vital staining of <u>E. coli</u> in the presence of dead bacteria .....                                                      | A7  |
| Table 8.  | The effect of bacterial and TTC concentration and incubation time on vital staining of <u>E. coli</u> in the presence of dead bacteria .....                                                      | A9  |
| Table 9.  | Growth of <u>E. coli</u> in continuous culture .....                                                                                                                                              | A10 |
| Table 10. | Growth of <u>E. coli</u> in continuous culture in minimal medium but complete medium introduced at 228 h .....                                                                                    | A11 |
| Table 11. | Growth of <u>E. coli</u> in continuous culture with minimal medium .....                                                                                                                          | A12 |
| Table 12. | Growth of <u>E. coli</u> in continuous culture with minimal and complete medium .....                                                                                                             | A13 |



|           |                                                                                                                                                                             |     |
|-----------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Table 13. | The effect of incubation time for 60 and 120 min in illustrating dehydrogenase damage of <u>E. coli</u> grown in the chemostat in minimal medium .....                      | A14 |
| Table 14. | Metabolic damage and death of <u>E. coli</u> grown in the chemostat(minimal medim)on exposure to 1.5 and 2.0 ppm chlorine .....                                             | A15 |
| Table 15. | Further evidence of metabolic damage and death of <u>E. coli</u> on exposure to chlorine at 32°C. Bacteria grown in the chemostat in minimal medim .....                    | A16 |
| Table 16. | Metabolic damage of <u>E. coli</u> with varying growth histories on exposure to 1 ppm chlorine .....                                                                        | A17 |
| Table 17. | Metabolic damage and death of <u>E. coli</u> grown in the chemostat on exposure to 1.5 and 2.0 ppm chlorine .....                                                           | A18 |
| Table 18. | A study of the effect of chlorine on glucose, malic and lactic dehydrogenases of <u>E. coli</u> .....                                                                       | 120 |
| Table 19. | The involvement of glucose, malic and lactic dehydrogenase in death of <u>E. coli</u> by chlorine .....                                                                     | 122 |
| Table 20. | The involvement of glucose, malic and lactic dehydrogenase in death of <u>E. coli</u> and <u>A. aerogenes</u> grown in batch cultures and exposed to 2.0 ppm chlorine ..... | 124 |
| Table 21. | A study of the influence of glucose and sodium lactate concentrations in substrate mixtures on the inhibition of dehydrogenase activity of <u>E. coli</u> .....             | 125 |
| Table 22. | The involvement of glucose, malic and lactic dehydrogenase in death of <u>E. coli</u> by chloramine-T ....                                                                  | A20 |
| Table 23. | Evidence that glucose may protect <u>E. coli</u> grown in minimal broth batch culture for 24 h at 32°C, from being killed by 2.0 ppm chlorine at 20°C and pH 7.2 ..         | A21 |
| Table 24. | Evidence that glucose may protect <u>E. coli</u> grown in the chemostat(minimal medium) from being killed by 2 ppm chlorine at 20°C and pH 7.2 .....                        | 129 |
| Table 25. | Survival of <u>E. coli</u> on exposure to 2.0 ppm chlorine at 32°C and pH 7.2. <u>E. coli</u> grown in the chemostat while operating on minimal broth .....                 | A22 |





## LIST OF FIGURES

|          |                                                                                                                                                                                                     |    |
|----------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Fig. 1.  | Typical break-point curve for chlorine and ammonia .....                                                                                                                                            | 39 |
| Fig. 2.  | Chemostat for continuous maintenance for bacterial population .....                                                                                                                                 | 63 |
| Fig. 3.  | The relationship between formazan production and bacterial concentration as illustrated by comparing turbidity of bacterial suspensions and optical density of solvent extract of formazan.....     | 82 |
| Fig. 4.  | The effect of TTC concentration on vital staining of <u>E. coli</u> with different culture histories .....                                                                                          | 85 |
| Fig. 5.  | The effect of TTC concentration on vital staining of <u>E. coli</u> maintained in the chemostat .....                                                                                               | 87 |
| Fig. 6.  | The effect of TTC concentration on vital staining of <u>E. coli</u> grown in batch culture in complete broth .....                                                                                  | 88 |
| Fig. 7a. | The effect of TTC concentration on vital staining of <u>E. coli</u> grown in batch culture in complete broth .....                                                                                  | 90 |
| Fig. 7b. | The effect of incubation time on vital staining of <u>E. coli</u> grown in batch culture in complete broth .....                                                                                    | 90 |
| Fig. 8a. | The effect of staining <u>E. coli</u> grown in complete broth in batch culture for 20 h at 32°C in the presence of dead bacteria .....                                                              | 92 |
| Fig. 8b. | The effect of bacterial concentration on vital staining with different TTC concentrations .....                                                                                                     | 93 |
| Fig. 9a. | The effect of bacterial, TTC concentration and time of incubation on vital staining of <u>E. coli</u> grown for 24 h at 32°C in complete broth batch culture in the presence of dead bacteria ..... | 94 |





|             |                                                                                                                                                                                                                                                      |          |
|-------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------|
| Fig. 9b.    | Amount of formazan produced by various concentrations of live bacteria with 500 ppm TTC. <u>E. coli</u> culture grown in complete broth batch culture for 24 h .....                                                                                 | 95       |
| Fig. 10a.   | The effect of bacterial and TTC concentration and incubation time on vital staining of <u>E. coli</u> maintained in the chemostat for 281 h. The culture was maintained in the chemostat with minimal broth at a dilution rate of 0.1/h at 32°C .... | 98       |
| Fig. 10b.   | Effect of TTC concentration on vital staining of the 100% live bacteria suspension .....                                                                                                                                                             | 98       |
| Fig. 10c.   | Effect of TTC concentration on vital staining of the 50% live bacteria suspension .....                                                                                                                                                              | 98       |
| Fig. 11.    | Growth of <u>E. coli</u> in continuous culture with minimal medium .....                                                                                                                                                                             | 103      |
| Fig. 12.    | Growth of <u>E. coli</u> in continuous culture in minimal medium but complete medium introduced at 288 h .....                                                                                                                                       | 104      |
| Fig. 13.    | Growth of <u>E. coli</u> in continuous culture with minimal medium .....                                                                                                                                                                             | 105      |
| Fig. 14.    | Growth of <u>E. coli</u> in continuous culture with minimal and complete medium .....                                                                                                                                                                | 106      |
| Fig. 15.    | The effect of incubation time for 60 and 120 min in illustrating dehydrogenase damage of <u>E. coli</u> grown in the chemostat in minimal medium .....                                                                                               | 111      |
| Fig. 16.    | Metabolic damage and death of <u>E. coli</u> on exposure to 1.5 and 2.0 ppm chlorine at 32°C and pH 7.2. <u>E. coli</u> grown in the chemostat in minimal medium .....                                                                               | 113      |
| Fig. 17a,b. | Metabolic damage and death of <u>E. coli</u> on exposure to 1.5 and 2.0 ppm chlorine at pH 7.2 and 32°C. <u>E. coli</u> grown in chemostat in minimal medium .....                                                                                   | 114, 115 |
| Fig. 17c.   | Metabolic damage and death of <u>E. coli</u> on exposure to 1.5 and 2.0 ppm chlorine at pH 7.2 and 32°C. <u>E. coli</u> grown in chemostat fed with complete broth ...                                                                               | 116      |
| Fig. 18.    | An example of metabolic damage of <u>E. coli</u> with varying growth histories caused by exposure to 1 ppm chlorine .....                                                                                                                            | 117      |



|          |                                                                                                                                                                                                     |     |
|----------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Fig. 19. | The involvement of glucose, malic and lactic dehydrogenase in death of <u>E. coli</u> caused by chloramine-T at 20°C and pH 7.2. <u>E. coli</u> grown in the chemostat in minimal medium .....      | 126 |
| Fig. 20. | Evidence that glucose may protect <u>E. coli</u> grown in minimal broth batch culture for 24 h at 32°C from being killed by 2 ppm chlorine at 20°C and pH 7.2 .....                                 | 128 |
| Fig. 21. | Difference in chlorine-resistance of <u>E. coli</u> grown in two runs in the chemostat while operating with minimal broth. Washed <u>E. coli</u> suspensions were exposed to 2.0 ppm chlorine ..... | 131 |



## LIST OF ABBREVIATIONS

|     |   |                                                                                                                                   |
|-----|---|-----------------------------------------------------------------------------------------------------------------------------------|
| CB  | - | Complete broth                                                                                                                    |
| EMB | - | Eosin methylene blue agar                                                                                                         |
| MB  | - | Minimal broth                                                                                                                     |
| NAD | - | Nicotinamide-adenine-dinucleotide                                                                                                 |
| NBT | - | 2,2' - di- <u>p</u> nitrophenyl - 5,5' - diphenyl -<br>3,3' - (3,5' - dimethoxy - 4,4' - diphenylene)<br>- ditetrazolium chloride |
| PMS | - | Phenazine methosulfate                                                                                                            |
| QAC | - | Quaternary ammonium compound                                                                                                      |
| TTC | - | 2,3,5 - triphenyl - tetrazolium chloride                                                                                          |





## INTRODUCTION

### DISINFECTION - GENERAL

The study of disinfection is still largely empirical. The novice, on delving into the voluminous literature on disinfectants and disinfection practice becomes aware of a sense of dissuasion to engage in unrestricted research in the field. The restriction in experimental approach stems from rather rigid rules set down in early procedures, such as the phenol coefficient test. It is apparent that many of the rules for testing practices cannot have been based on a good understanding of the disinfection process since little is known about this even today. Many of the early tests attempted to simulate conditions under which a disinfectant would have to act, by including organic material in the test. This practice further complicated a study of mechanisms of disinfectants, since it did not allow the experimenter to observe the interaction of the lethal agent and bacteria, but simply revealed what effect a chemical might have on a bacterial population in the presence of competitive and protective materials. This problem was recognized by Eddy (1953 a,b) and Eddy and Hinshelwood (1953) in an excellent series of papers on death rate of bacterial populations but their conclusions on mechanisms of disinfection lack the direct support of experimental results. Thus after more than a half a century of intensive research on the mechanisms involved in resistance of bacteria to disinfectants Moyed (1964) stated:



"The discussion of resistance will be limited, for the most part, to those cases in which the inhibition has a single predominant site of action. Single step mutants with resistance to multisite metabolic inhibitors such as fluoride, cyanide, dinitrophenol and sulfhydryl reagents, or highly reactive chemical disinfectants are rare and difficult to analyze, thus, definitive studies of the biochemical mechanism for resistance to these agents are lacking."

The terms disinfection and disinfectants are often loosely defined, as for example, by Warner and Maniar (1965) who define disinfectants as:

"examples of a large group of widely used and important substances ranging from antibiotics to food preservatives which have in common the property of antimicrobial action."

Whereas the author is inclined to agree with this definition, it should be pointed out that the terminology followed throughout this paper will conform to the suggestions of Sykes (1965) and Reddish (1957) who regarded a disinfectant as a chemical which destroys micro-organisms. These chemicals are usually used on inanimate objects and are bactericidal or bacteriostatic to the vegetative stage of the micro-organism. These agents may not inactivate resistant spores which should be regarded as a special problem of





disinfection (Sykes, 1962). Davis (1960a) makes a strong case for the use of proper terminology in the field. He points out that "disinfection" is not "sterilization". Sterilization is an absolute term, indicating the complete absence of all forms of life, whereas disinfection indicates a reduction of micro-organisms to a "safe" level (or to prevent infection). This may mean the destruction of pathogens only in the medical sense, or the killing of micro-organisms causing spoilage in the food industry, or the destruction of a certain arbitrary fraction of bacteria present in a solution, as in suspension tests for evaluating disinfectants. A bactericide kills bacteria, but not necessarily bacterial spores. The killing effect may be due to poisoning of the bacterial metabolic system or may be caused by actual lysis of bacteria; however, a bacteriostatic agent is never lytic. These agents prevent multiplication of bacteria and result in a state of suspended animation.

The literature on disinfection abounds with observations on the effects of disinfectants on bacterial populations; however, papers dealing with the mechanisms involved are few. This is especially true with reference to chlorine and chlorine-containing compounds which are probably still the chemicals most commonly used as industrial disinfectants. Large amounts of chlorine gas and hypochlorites are used for treating sewage and water; however, the disinfection of food processing equipment and of eating utensils are other important applications of chlorine. It is therefore surprising, as pointed out by Sykes (1965), that relatively little has been published on the death





rates of bacteria under the influence of chlorine, and even less appears to be known with certainty about mechanisms involved in death or resistance of bacteria to chlorine.

The need for new approaches in problems involving testing the effectiveness of chemical disinfectants was recently emphasized by Sykes (1962). In his address as President of the Society for Applied Bacteriology he stated:

"And now what of the future? In terms of antiseptic evaluation, apart from a certain desirable rationalization of some of the methods, there seems to be little difficulty. But with disinfectants the situation is quite different, and in this context let me make three categorical statements:

1. There is no future for the phenol coefficient type of test.
2. An entirely fresh and untrammelled approach to the problem is needed.
3. In the meantime there is probably little point in trying to improve the existing tests. Such improvements as can be envisaged are unlikely to reconcile the divergent results often found between laboratories, although there is always the possibility that they might expose the reasons for them.

In passing one might draw attention to a little noticed defect in the technique for assessing the survival of organisms by plate counts. Before a culture can be used in a test it must be first grown for a period, usually in an incubator at 37°C for 24 hours. It is



then cooled in a water bath to the temperature of the test and subjected to a major assault by the disinfectant. After this the injured cells are diluted in broth, saline, or some other fluid, probably at a temperature slightly different from that of the test. Then they are heated practically instantaneously to 40°C or so when inoculated into the molten agar, after which they are allowed to cool while the agar sets; lastly they are transferred to their final resting place in the incubator, again at 37°C. No wonder few of them survive."

If we agree with a specialist such as Sykes that all is not known about how disinfectants kill bacteria and that there exist unsolved problems with reference to evaluating disinfectants, and that the control of micro-organisms in our environment with disinfectants is still an important aspect of the field of bacteriology, then it becomes quite apparent that further research in the field of disinfection is justified.

When studying the action of a disinfectant on a bacterial cell it is important to realize that the inhibited cell is in a dynamic state; this means that the primary interaction of the lethal agent and the cell triggers many secondary reactions which may eventually result in autolysis of the cell. Thus Oginsky (1953) noted that the inhibition of oxidative enzymes by streptomycin



appeared to be secondary to death; however, if it is true, as appears from the literature, that chlorine kills bacteria by binding to aromatic amino acids and SH-groups of enzymes or other proteins, it should be possible to decide whether these are primary reactions if one could observe that changes in enzyme activity also result in changes in chlorine resistance. It is therefore probable that the active centers of enzymes might be most susceptible to inactivation by lethal agents.

It appears certain that death is not due to cell lysis or nonspecific protein denaturation since concentrations of disinfectant causing rapid death of most vegetative bacteria are too small for this (Knox et al. 1948). In the past, bactericidal powers of chlorine have frequently been expressed in terms of the minimum concentration producing a kill of 99% or greater in a given time under specific experimental conditions; however, recent reports on significant differences in chlorine-resistance of E. coli caused by physiological differences (Milbauer and Grossowicz, 1959a, b) and the illustration of a chlorine-resistant mutant of this micro-organism by Farkas-Himsley (1964) also suggested that further research was warranted on bacterial death by chlorine.

It has been generally accepted that the shape of survivor curves where chlorine or other disinfectants act on a bacterial population, depends on the rate of kill. Thus a straight line relationship could be illustrated with high concentrations of







disinfectants, whereas sigmoid death curves were frequently observed when the killing rate was slow as a result of less severe disinfection conditions.

In the present work an attempt to extend observations on bacterial death curves resulting from the action of chlorine on washed populations of E. coli by using membrane filters to recover and concentrate small numbers of survivors in the later stages of disinfection experiments, met with failure because dead bacteria present in the suspension clogged the filters. This meant that quantities greater than 10 ml required a long time to be filtered through the membrane. Another difficulty experienced was that no agreement could be obtained between agar pour plates or roll tube counts and the colony counts on membrane filters. Frequently, confluent growth was observed on filters where the pour plates suggested that countable numbers should have been expected. It was considered that these results were not caused by contamination from prolonged vacuum treatment since the air was drawn through cotton filters. The only reasonable explanation was that many chlorine-injured bacteria succumbed when hot agar (45 - 50°C) was introduced into the plates. This was suggested by Sykes (1962) and Stapert et al. (1962) who presented evidence that organisms in a nutritionally poor environment and at low temperature frequently cannot tolerate the physical stress caused by an increase in temperature of liquid agar. All work reported here was therefore done by using the modification of the spot plate technique described by



Gaudy et al. (1963) for estimating viable bacteria.

Early after changing to this technique an observation was made which greatly influenced the direction of this investigation. E. coli not exposed to chlorine developed into uniform colonies on the surface of agar plates; however, E. coli surviving exposure to chlorine frequently formed small colonies or colonies varying considerably in diameter. If the washed bacteria were incubated with a small amount of glucose before exposure to chlorine, no apparent colonial differences could be noted. Since most carbohydrates do not appear to react with chlorine (Allen and Brooks, 1952), it was considered that this might be an effective protective mechanism. It was felt that the mechanism involved might be similar to the substrate protection of glucose dehydrogenase as illustrated by Sadoff et al. (1965).

It now appeared possible to the author that bacteria may not be killed by disinfectants by the random interaction of a few sensitive regions of the cell and the lethal agent, but that death of the bacterial cell could be caused by the simultaneous or sequential inactivation of sufficient enzymes and/or other cell proteins to make it no longer possible for the bacteria to repair the damage and to multiply. There exists a large amount of circumstantial evidence to support this view:

1. Bacteria normally do not acquire resistance to disinfectants, although a few cases have been reported; however such resistance is not retained for long periods if the micro-





organisms are subcultured in the absence of the disinfectant (Cook, 1960; Ortenzio et al., 1949). Eddy and Hinshelwood (1953) explained that the resistance of bacteria to disinfectants depends on the immediate condition of the cells at the time when the culture is exposed to disinfectants. Experience rules out the possibility that more resistant members of a population are mutants since subculture of late survivors in disinfectant experiments usually give rise to a population of similar resistance to the parent population. Complete reversion during a single subculture is most unlikely in the opinion of Eddy and Hinshelwood.

2. "Adaptive adjustments" may occur in a dying population of bacteria especially in the presence of slow acting agents, when certain nutrients, such as glucose, are present as reported by Eddy and Hinshelwood (1953). Perhaps this is substrate protection of susceptible enzyme sites from damage by the lethal agent.

3. The direct involvement of protein and specifically enzyme SH-groups in increasing chlorine-resistance of E. coli as illustrated by Milbauer and Grossowicz (1959 b).

As a result of the above considerations it was felt that further information was needed on the changes which occur in bacteria when they die due to the action of a lethal agent such as chlorine. The present work is an attempt to furnish experimental data which would show that death of bacteria due to the action of chlorine is accompanied by the destruction of a number of different metabolic





sites. It was felt that the illustration of damage by chlorine to a number of different enzyme systems, occurring simultaneously but independently, while bacteria die due to the action of chlorine, would lend support to the theory first put forth by Eddy (1953). This theory proposed that death of the bacterial cell involves not only progressive destruction of a number of essential cell activities, but may also be conditioned at some stage by a random combination of various independent events.

Vital staining with TTC was investigated as a means of demonstrating enzymic damage brought about by chlorine treatments. The reduction of TTC to formazan has been widely accepted as a means of measuring the activity of numerous dehydrogenase systems in biological materials. Since dehydrogenases with the ability to reduce TTC occur at different locations on the bacterial cell it was felt that a TTC test could be used to illustrate localized damage by the use of specific substrates. Perhaps also by the use of complex substrates the test could be used as a rapid method for estimating generalized damage by a disinfectant to a bacterial population.

Jensen et al. (1951) investigated the usefulness of TTC in illustrating a number of dehydrogenases and in the present work it was decided to use glucose, lactate, and malate to study the influence of chlorine on the utilization of these three carbohydrates. These workers suggested that the use of the above substrates would illustrate the dehydrogenase mediated conversions of glucose to



gluconate, lactate to pyruvate, and malate to oxalacetate; however it must be admitted that other reactions may also be involved. Thus in the present work the terms glucose, lactic and malic dehydrogenase are used to indicate oxidative utilization of the substrates in question. A similar approach was used by Knox et al. (1948) and Gould et al. (1957) and this appears to be compatible with the description of dehydrogenases as enzymes responsible for the oxidation of a number of organic compounds including glucose, lactate, malate as given by Thimann (1963).

The effect of pre-incubation of bacteria with glucose, a compound which does not appear to react with chlorine, was studied to see if protection of enzyme systems by their substrates might result in reducing the effectiveness of chlorine in killing a bacterial population. Occurrence of substrate protection would thus implicate the destruction of enzyme proteins as a major mode of action of chlorine.



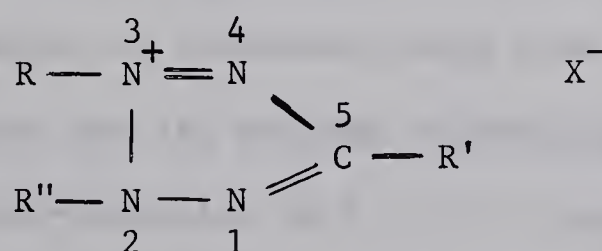
## REVIEW OF SELECTED LITERATURE

### TETRAZOLIUM SALTS

While investigating the influence of additional N-atoms near the charged N-atom of a quaternary ammonium compound, Kuhn and Jerchel (1941 a, b) noted that the colorless, water soluble, toxic tetrazolium salts could be reduced by bacteria, yeasts, and germinating seeds to the water insoluble, strongly colored less toxic formazans. The phenomenon of tetrazolium reduction and formazan deposition has been widely accepted as an indication of enzyme activity and cell viability in the field of biology. The tetrazolium salts are of special interest as oxidation-reduction indicators in biological systems since the red formazan is stable to oxygen and it is possible to work under aerobic conditions. However, since these compounds are a type of quaternary ammonium compound (Lawrence, 1950) it is important to appreciate their properties as protein denaturants and disinfectants.

### The Chemistry of Tetrazolium Salts

Tetrazolium salts are quaternized tetrazoles, with the general structural formula described by Lau (1964):



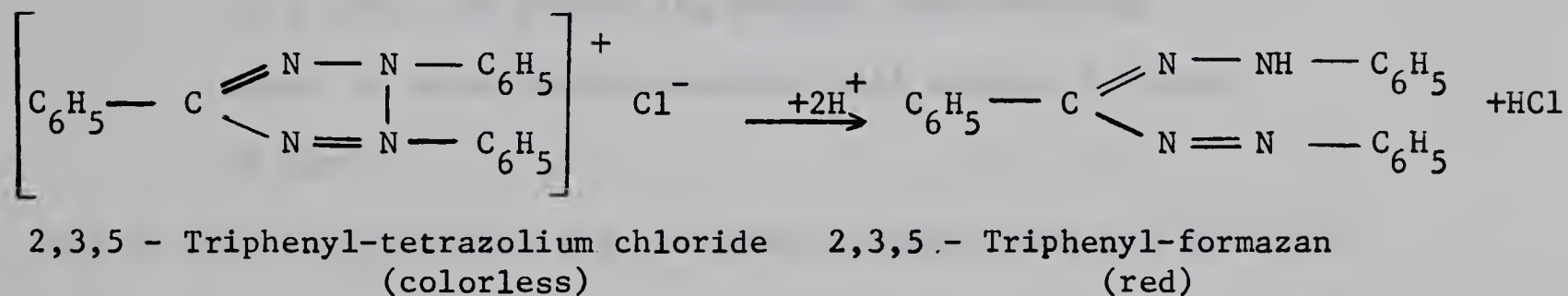






R and R'' are aryl groups on all tetrazolium salts at present investigated. A wide variety of C - substituents (R') have been reported (Nineham, 1955) and X can be any anion; however, halogen derivatives are common.

Tetrazolium salts are rather large molecules with molecular weights ranging from 300 to 800. The salts are pale yellow in color and may darken on exposure to light. Ultraviolet light may convert them to the colorless phototetrazolium salts. The tetrazolium salts are soluble in water and most alcohols. On reduction they form the rather stable formazans (British Drug Houses, undated) as follows:



While the reduction of tetrazolium salts is accomplished in many ways, the formazans are oxidized to tetrazolium salts only with difficulty, and by certain oxidizing agents, such as mercuric oxide or lead tetra-acetate. The formazans are insoluble in water, but can be dissolved in chloroform, acetone and most other organic solvents. The colors of formazans range from red to purplish-black. Triphenyl-formazan has its maximum absorption at about 490 mμ and the apparent redox-potential of 2, 3, 5 - triphenyl-tetrazolium chloride was found to be about -0.08 volt by Jerchel and Möhle (1944).



Reduction of tetrazolium salts to their formazans by sugars (Mattson and Jensen, 1950), ascorbic acid, cysteine and glutathione (SH) occurs only in an alkaline solution ( $\text{pH} > 9$ ). In a medium of a pH lower than 6, triphenyl-tetrazolium chloride is chiefly reduced to a colorless product. Light seems to influence enzymatic as well as non-enzymatic reduction of triphenyl-tetrazolium chloride. Both types of reduction appear to result in red formazan mainly in the light; however, non-enzymatic reduction in strongly alkaline solution occurs even in the dark. Jámboř (1954) stated,

".... the redox-potential of formazan formation, contrary to other redox-indicators, cannot serve as a basis for predicting whether some reducing agent of known redox-potential will produce formazan or not."

This is apparently because the insoluble formazan does not allow for the establishment of a redox-equilibrium. The actual potential will be much more positive than the normal potential. This explains why succinic dehydrogenase, which has a much more positive redox-potential (a redox-potential of 0.00 volt at pH 7.0 has been reported by Jensen et al., 1951, for the succinic dehydrogenase mediated conversion of succinate to fumarate), will reduce triphenyl-tetrazolium chloride.





### Biological Applications of Tetrazolium Salts

Since the initial discovery by Kuhn and Jerchel (1941 b) of the vital staining properties of tetrazolium salts, these compounds have been widely used for the elucidation of many biochemical processes in micro-organisms and tissue sections. Two excellent reviews in English (Smith, 1951) and German (Ried, 1952) give accounts of the immediate acceptance of these compounds as vital stains. The present review will deal with tetrazolium salts (especially 2, 3, 5 - triphenyl-tetrazolium chloride) as a tool for studying damage inflicted on E. coli by disinfectants.

It was hoped that the lethal action of disinfectants could be demonstrated by tetrazolium salts in the following four ways:

1. Illustration of damage to all enzymes which have the ability to reduce TTC by measuring the decrease in formazan production of bacteria growing in a complex medium.
2. Illustration of specific enzyme damage caused by disinfectants by supplying pure substrates.
3. Illustration of enzyme damage caused by quaternary ammonium compounds by the use of large amounts of tetrazolium salt as the lethal agent.
4. By measuring protein-bound sulfhydryl and disulfide groups with the alkaline tetrazolium salt method.





### Enzymic Reduction of Tetrazolium Salts

Mattson et al. (1947) noted that the reduction of tetrazolium salt in plant and animal material at between pH 6.5 and 8.5 involved enzymes. They showed that heating of such material to 82°C caused loss of the ability to form formazan, whereas freezing had no influence. They postulated that NAD linked glucose dehydrogenase was involved. Siegert (1949) suggested that reduction sites might be located with tetrazolium salts in the bacterial cell. Kun and Abood (1949) appear to be the first to use tetrazolium reduction for a colorimetric determination of succinic dehydrogenase. They showed that aerobic and anaerobic incubation eventually yield the same amounts of formazan, although anaerobic incubation may lead to faster initial reduction. Since these early observations, the reduction of tetrazolium salts has become a widely accepted method for demonstrating dehydrogenase activity in biological systems (Raabo, 1963) and is now widely used in histological studies. Of particular interest is the fact that dehydrogenases are located at many sites in the metabolic system of bacteria. This means that the use of tetrazolium salt allows the illustration of multiple enzyme damage by such agents as disinfectants.

The exact mechanism by which tetrazolium salts are reduced by pyridine nucleotide dehydrogenases is at present being investigated (Hashimoto et al., 1964). It is certain that tetrazolium salts are good electron acceptors and are reduced because of the



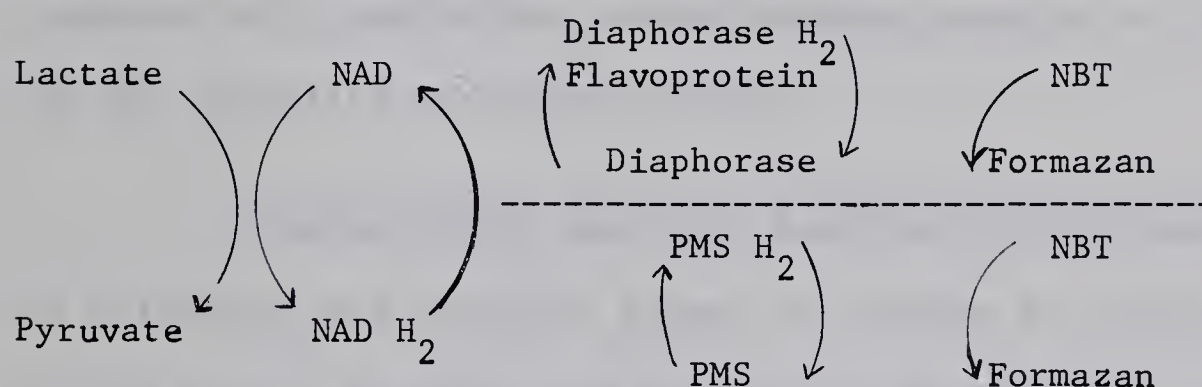
oxidation of various substrates by dehydrogenases. In natural systems the demonstration of dehydrogenases by the tetrazolium method appears to be limited by the final step of hydrogen transfer from the substrate of the dehydrogenases to the tetrazolium and probably flavoproteins mediate formazan formation. The use of flavoprotein substitutes, such as phenazine methosulfate (PMS) has allowed a proposed mechanism for enzymic reduction of tetrazolium salts:



Phenazine methosulfate has been used as a non-enzymatic hydrogen transfer mediator from reduced pyridine nucleotide to tetrazolium, that is as a substitute for the flavoprotein component in the hydrogen transfer mechanism, in the study of lactic, succinic, isocitric, malic and glucose dehydrogenases in tissues. Results of the effect of phenazine methosulfate on the formation of formazan or activity of dehydrogenases are conflicting. Thus Van Wijhe et al. (1963) found that phenazine methosulfate results in a partial suppression of 2,2' - di-p-nitrophenyl - 5,5' - diphenyl - 3,3' - (3,5' - dimethoxy - 4,4' - diphenylene) - ditetrazolium chloride (NBT) reduction by lactic dehydrogenase in the red fibers of skeletal muscle of the rat whereas Hess and Pearse (1961) noted that phenazine methosulfate had no effect as a substitute as electron carrier in the illustration



of various dehydrogenases in similar tissues. Nachlas et al. (1960) stated that tetrazolium salts are reduced by the enzymic oxidation of succinate both with and without phenazine methosulfate. In the former situation the electrons are transferred from enzyme to phenazine methosulfate to tetrazole, but the pathway involved without phenazine methosulfate (about one-third the rate) remains to be determined. Van Wijhe et al. (1963) have proposed a scheme relating the two possibilities for lactic dehydrogenase:



The papers of Brodie and Gots (1951, 1952) favor the importance of flavoproteins in the reduction of tetrazolium salts. With phosphoglyceraldehyde dehydrogenase isolated from yeasts, these authors showed that the presence of tetrazolium salts did not interfere with the reduction of NAD, as shown by spectrophotometric measurements at 340 mμ; however, no formazan was formed unless the flavoprotein, diaphorase, was added. On addition of diaphorase, reduction of tetrazolium salts occurred aerobically as well as anaerobically. In the second paper this principle was expanded to show that dehydrogenases from yeasts and E. coli depended on flavoproteins as electron carriers in the reduction of tetrazolium salts.





Alkaline Tetrazolium Salt as a Method of Illustrating  
Protein-bound Sulfhydryl and Disulfide Groups

Since Milbauer and Grossowicz (1959 b) implicated the amount of available protein-bound sulfhydryl groups, that is "enzymatic SH", in chlorine resistance of E. coli, and since SH groups may lead to non-enzymatic reduction of tetrazolium salts at alkaline pH, thus possibly leading to apparent greater dehydrogenase activity as measured by tetrazolium salt, it is important to consider this problem when using formazan formation as an indicator of cell viability or enzyme activity.

Findlay (1955) described a method for the determination of sulfhydryl and disulfide groups of proteins by alkaline tetrazolium salts. He noted that neotetrazolium chloride is more suitable for sulfhydryl determinations because color intensity and formazan stability were superior for this salt than for triphenyl-tetrazolium chloride. The procedure depends on the fact that sulfhydryl groups reduce tetrazolium salts at alkaline pH. When a pH over 11 is used both sulfhydryl and disulfide groups are demonstrated because disulfide groups are reduced. To allow the illustration of these groups separately, Deguchi (1964) used NBT at pH 8.4, which is considered not to reduce disulfide groups. He also illustrated that glucose does not reduce this salt below pH 9.2. The inclusion of sulfhydryl blocking agents such as iodoacetate, and disulfide splitting agents such as potassium cyanide, allowed the demonstration



of disulfide and sulfhydryl groups in proteins.

Thus non-enzymic reduction of tetrazolium salt, except for the reduction by direct sunlight or bright daylight, appears to be limited to alkaline conditions. Many tetrazolium reducing compounds such as free cysteine and reducing sugars are water soluble and may be removed from cells during washing; however, the inclusion of sulfhydryl blocking agents is not possible in measuring dehydrogenase activity, since they may have an inhibitory effect on sulfhydryl-related dehydrogenases.

The Bactericidal or Bacteriostatic Properties of Tetrazolium Salts and Other Quaternary Ammonium Compounds

Kuhn and Jerchel (1941 b) showed that the bactericidal properties of quaternary ammonium compounds were a result of their protein denaturation properties. They noted that bacteria were killed because vitally important proteinaceous cell components of bacteria were destroyed. They could not show whether the destruction of cell proteins occurred solely at the bacterial surface, because it was not possible to show whether quaternary ammonium compounds could penetrate the bacterial cell rapidly. It could be shown, by extraction with xylol that formazan, formed from tetrazolium salt by yeasts, was deposited inside the cell. Experiments with lactic acid bacteria (Streptobacterium plantarum) also confirmed intracellular deposition of formazan; however, staphylococci showed internal and external deposition of formazan. This observation has





more recently been confirmed by Eidus et al. (1959), who noted that TTC is taken up by both Gram-positive and Gram-negative bacteria which build up large irregular deposits of formazan inside the cell. Gram-positive organisms also deposit formazan outside the cell wall (Korn and Kushnarev, 1965).

Jerchel (1942) showed that 2, 3 - diphenyl - 5 - undecyl-tetrazolium chloride had bactericidal properties towards lactic acid bacteria (Streptobacterium plantarum) similar to those of "Zephirol" a commercial quaternary ammonium compound. He further showed that this disinfectant and a number of tetrazolium salts are similarly bactericidal because of their suppression of glycolysis in the lactic acid bacteria studied.

It now becomes increasingly apparent that a knowledge of the effect of surface active compounds on proteins and of the bactericidal properties of these compounds is of paramount importance, when one considers the use of tetrazolium salts for the determination of dehydrogenase activity or vital staining of bacteria.

The fact that formazan is deposited inside the bacterial cell still does not preclude the bactericidal effect being a result of disruption of the cell membrane as demonstrated by Hotchkiss (1946). He noted that bactericidal surface active agents often have no chemical relationship except some similarity of the hydrophobic-hydrophilic structure. He stated that combinations of these compounds with oppositely charged components to the bacterial surface



is the prime factor in their bactericidal efficiency. Damage to the cellular membrane results in leakage of soluble nitrogen and phosphorus from the cell. It is suggested that disorganization of the cell membrane thus caused may lead to pronounced retardation of the bacterial metabolism. That is to say suppression of glycolysis and respiration as observed by Miller and Baker (1940) and Baker et al. (1941 a, b) is caused by the action of surface active agents on bacteria and leakage of metabolites into the medium, rather than penetration into the cell and interference with the enzymes involved in glycolysis and respiration.

Gale and Taylor (1946, 1947) noted that Streptococcus faecalis cells release lysine and glutamic acid after being exposed to antibiotics, surface active agents and phenol; however, they also noted that phenol causes denaturation of lysine decarboxylase. Salton (1951) presented further evidence that leakage of 260 mμ - absorbing material and other internal cell constituents such as free purines, can be related to the proportion of cells killed when E. coli, Staphylococcus aureus and other bacteria are treated with quaternary ammonium compounds. He noted that more surface active agent is absorbed than would be required to saturate the cell surface, suggesting that both surface and internal components of the bacterial cell can be titrated with cationic detergents. Supporting electron micrographs suggested a contraction of the cytoplasm from the cell wall, permeability alterations of the





membrane and complete stripping of the cell wall by high concentrations of surface active agent.

In spite of all the evidence that cell disruption by surface active agents causes bacterial death, much evidence has been presented that enzyme inactivation is also involved. Valko (1946) noted that E. coli could be saturated throughout with dimethyldodecyl benzyl ammonium chloride when the latter was present in a dilution which kills in 5 min. This suggested that one or more mechanisms, such as denaturation and precipitation of proteins, cleavage and inactivation of protein complexes, particularly enzymes, and lysis of cells are involved in lethal action of surface active agents on bacteria. Roberts and Rahn (1946) demonstrated complete inactivation of cell dehydrogenase systems by lethal concentrations of a number of disinfectants, including "Zephiran". Similarly Knox et al. (1949) showed that E. coli possesses presumably essential enzymes which can be inactivated by quaternary ammonium compounds in vivo and in vitro by bactericidal concentrations. They demonstrated that lactic oxidase is susceptible to cationic surface active agents and noted that enzymes which have been inhibited by detergent cannot be reactivated by removal of the detergent, by additional substrate, or by any known cofactor. There is no indication of prosthetic group separation or protein denaturation in such an inhibited enzyme.





Stedman et al. (1957) showed that concentrations of quaternary ammonium compounds required to inactivate partially the glucose oxidizing system of Serratia marcescens are much lower than those required to effect significant cell wall destruction. This view is most ably supported in a recent paper by Cox (1965) who showed that quaternary ammonium compounds rapidly penetrate the bacterial cell. The largest portion of the lethal agent appeared to become associated with the bacterial protoplast, whereas a small amount appeared to associate with the cell-wall fraction as illustrated by measuring a lysozyme digest of the cells. Using a C<sup>14</sup> labelled QAC it was found that the association of the QAC with the cell wall material was independent of increasing external QAC concentration.

It is now apparent that the most probable reason that surface active compounds, including tetrazolium salts, are bactericidal and bacteriostatic is caused by interference with enzyme systems. An interesting aspect of the tetrazolium salts is that they may inhibit, at sufficiently high concentrations, the very enzymes which reduce them to formazans. This suggested that tetrazolium salts should be useful for investigating bactericidal and bacteriostatic mechanisms. It appears that at low concentrations the salts are subject to substrate-enzyme kinetics with an increasing amount of formazan being produced as the concentration of tetrazolium salt increases; however, at a certain



enzyme to tetrazolium salt ratio the reduction to the less toxic formazan can no longer occur and suppress the inactivating properties of the tetrazolium salt, and formazan production will decrease with further increases in tetrazolium salt concentrations. It appeared worthwhile to attempt to illustrate this self monitoring property of tetrazolium salts and some data regarding this will be given later.

The Use of TTC as an Indicator of Bacterial Damage  
by Chemical Agents

Wallhäusser and Rippel-Blades (1950) noted that antagonisms between organisms growing on the same plate may often be missed because no inhibition zone is noticed since bacteria are generally not lysed when they die as a result of the influence of a lethal agent. They showed that no inhibition zone could be noted when Aspergillus terreus was inoculated on to a surface plate of Bacillus mycoides. However after the plate had been flooded with triphenyl-tetrazolium chloride it became apparent in a few minutes that the bacteria had been killed around the fungal colony. Wallhäusser (1951 a) used the above procedure to illustrate antagonisms among micro-organisms in a natural microflora, and noted that age of the culture was often an important factor in formazan formation; however, Stolp (1952) pointed out that the ability to reduce tetrazolium salts is lost in aging Bacterium coli cultures could be restored by the addition





of glucose. Usdin et al. (1954) markedly improved the sensitivity of bioautography by including the tetrazolium salt, whereas Wright and Tramer (1961) showed that bacteria would not reduce tetrazolium salt in milk containing traces of antibiotics. Kopper (1952) reported that a straight line relationship existed between numbers of bacteria and formazan production when bacterial concentrations ranged around  $10^{10}$  cells/ml of E. coli. This fact has been used for rapid estimation of the number of viable bacteria in BCG (bacille Calmette Guérin) vaccine for tuberculosis (Eidus et al., 1958). Furthermore these workers observed that tetrazolium salts tend to inhibit bacterial multiplication. They also showed that picric acid could be used to stain dead organisms after live organisms had been stained with tetrazolium. However attempts by the present author to use the ratio of dead to live bacteria thus obtained, as a rapid guide to assess the degree of damage done to a bacterial population on exposure to disinfectants, met with no success.

At least three papers have been published suggesting or illustrating the use of tetrazolium salts for estimating the effectiveness of disinfectants. Wallhäusser (1951 b) was the first to write that a tetrazolium test could be used to ascertain both killing time and reduction in numbers of survivors. He used Staph. aureus as the test organism and formazan was extracted with ethanol and amyl alcohol for colorimetric determination



of the degree of damage; however, the paper gives a rather limited amount of data.

Murányi (1959) described a very simple test for evaluating disinfectants. A drop of buffer, a drop of disinfectant solution and two drops of a washed suspension of E. coli containing  $10^{10}$  cells/ml were mixed in the depression of a spot plate. After 10 min a drop of glucose solution and a drop of tetrazolium salt solution were added. The spot plate was incubated for 10 min at 37°C and the amount of formazan present was used as an indication of survivors. The author concluded that this micromethod could furnish useful information about the effectiveness of any disinfectant in 30 min.

The original test by Wallhäusser (1951 b) was modified by Sunila and Parmala (1955) to estimate phenol coefficients of QACs. The test organism was Proteus vulgaris grown in nutrient broth. After the bacteria had been exposed to the germicide for 5 min, tetrazolium chloride was used to estimate the damage done by the disinfectant. Formazan was extracted from the cells by acetone and a colorimetric estimation of the formazan was accomplished after it had been dissolved in n-butanol. The authors reported that phenol coefficients obtained in this manner for a number of QACs did not differ from results reported in the literature elsewhere, and the test appeared to satisfy most of the following criteria for a method for evaluating disinfectants: 1, simplicity;





2, inexpensiveness; 3, accuracy; 4, reproducibility; 5, usefulness over a range and 6, relation to the service the product is to perform. The authors noted that the accuracy of the test was not good, and it should be pointed out that no inactivators for disinfectants were used and no attempts were made to standardize either substrate or bacterial concentrations. It appears that experimental conditions must be controlled more carefully and in the present work it is shown that the accuracy and reproducibility of a tetrazolium test can be greatly improved by stabilizing substrate concentrations and the numbers and physiological condition of the test organism. It was obvious that further research was justified to establish the usefulness of the above procedure, and it is interesting to note that the above three papers were not mentioned in the recent edition of the textbook dealing with disinfection by Sykes (1965).

#### DYNAMICS OF DISINFECTION

In 1897 Krönig and Paul showed in their classical method for the quantitative study of disinfectants, that disinfection does not take place instantaneously (see Wilson and Miles, 1964). Nevertheless it is commonly accepted that Chick (1908; 1910) was one of the first to study the disinfection process in a quantitative manner. She found, during the process of disinfection of anthrax spores by chemical disinfectants (mercuric chloride and phenol respectively), that the concentration of spores remaining alive





varied logarithmically with time, and that the number of spores destroyed per unit of time was proportional to the number present in that volume of the medium at that moment. This observation led to the fact that points, obtained when plotting the logarithm of the number of bacteria surviving against time of exposure to the lethal agent, lay on a straight line. These curves appeared similar to those obtained when plotting the progress of a unimolecular reaction and accordingly the disinfection process may be expressed by the following equation:

$$\frac{1}{t_2 - t_1} \log \frac{M_1}{M_2} = K$$

where  $M_1$  and  $M_2$  are the numbers of surviving bacteria after times  $t_1$  and  $t_2$ . This equation represents the velocity of a unimolecular reaction, such as the decay of radio-active substances, and is often spoken of as the logarithmic law. If values for  $\log M_2$  are plotted against time the resulting graph is a straight line. It would however be extremely surprising if disinfection processes were to follow such a simple mathematical law since it is obvious that the process not only involves the interaction of the disinfectant with a single cell constituent, but also a number of complex physio-chemical phenomena such as variability in resistance of individual cells in the population, penetration of the disinfectant and interaction of the disinfectant with critical sites within the bacterial cell. Chick, one of the controversial writers in this field,



noted (1908) that:

"The process, although really involving two 'reagents', follows the law of a unimolecular reaction, because the second reagent, the disinfectant, is present in so great an excess, comparatively, that its concentration may be regarded as unaltered during the process. ....Experiments with cultures of Bacterium paratyphosus show a departure from this simple law, the reaction velocity diminishing during disinfection more rapidly than is accounted for by the fall in number of the surviving bacteria. This was the case with each of the three types of disinfectant used. This divergence is due to differences in resistance between individuals of the various ages contained in the culture".

She also showed that the younger individuals of a culture possess a higher resistance to disinfectants, whereas it is now commonly accepted that bacteria exhibit more resistance to lethal conditions on ageing. The fact is that exponential curves from disinfection experiments can be expected only when the disinfectant is present in excess and the rate of disinfection is sufficiently rapid to make detection of divergences from the straight line impossible (Jordan and Jacobs, 1944 a). One of the dangers of accepting the logarithmic death rate, according to Phillips and Warshowsky (1958), is that experimental data frequently fit a





semilogarithmic plot within experimental error and since it is such a convenient way of treating disinfection data, scant attention is usually paid to cases of poor fit, or to the fact that no good rationale has been developed as to why such a simple mathematical law should logically define the disinfection process. In some cases small but significant numbers of surviving organisms are found after extended disinfection times, where an extrapolation of the exponential death curve would have indicated a very low probability of any living organisms in volumes tested. In fact it has recently been shown by Jacobs (1960) that with an extended disinfection with critical lethal concentrations an actual recovery of some of the cells may occur. Bean and Walters, (1961) interpret the slight increase in viable organisms as probably caused by the change of the environment because of release of cellular material when killed cells lyse.

The occurrence of a straight disinfection curve was questioned by many workers as early as 1908 (see Wilson and Miles, 1964) and non-exponential curves have been reported more frequently than those confirming the logarithmic law. Thus Withell (1942) suggested that three general types of time-survivor curves may exist: a) those which are sigmoid in shape; b) those which are exponential; and c) those which show a lag phase followed by an exponential response. Eddy (1953 b) reported a fourth type of survivor curve which might be described as an exponential response followed by a decline in death-rate in the later stages of exposure.



What can be learned from time-survivor curves? Probably the first thing that should be noted is that time-survivor curves reduce the disinfection process into an "all-or-nothing" response. That is to say that damage to a part of the metabolic machinery or structure of the cell which may be repaired during the incubation period is completely disregarded by the time-survivor approach to disinfection. The author feels that survivor counts alone are not too meaningful unless they are supported by evidence such as inactivation of enzymes or proteins in general, or other changes in the cell. Thus the monomolecular theory of cell destruction leads to the following implications:

1. The resistance of individual bacteria composing a population under test is essentially uniform.
2. Death of a cell is due to one or very few random events between the lethal agent and a sensitive, critical region of the cell.
3. The action of the disinfectant is non-cumulative and that the cell is unaffected until the lethal event occurs.

Clark (1933) pointed out that the mere statement of these postulates appears absurd and becomes impossible to discuss when related to other biological factors. Thus an investigation of the literature at that time revealed that of 154 reports on the action of lethal agents on bacteria only 32 reports showed a constant rate in





keeping with the monomolecular theory. The concept of death being caused by a few molecules of disinfectant is contradictory to activities of living materials which always display variation in biological properties.

The obvious alternative to the monomolecular theory is to assume that the length of time a bacterium can survive a bactericide is proportional to its resistance. Thus various types of sigmoid time-survivor curves may result from disinfection experiments. Generally speaking, sigmoid curves become steeper, and even approach exponential curves as death of the population becomes accelerated. Withell (1942), Jordan and Jacobs (1944 a) and Jordan et al. (1947) indicated that disinfection curves normally depart from the logarithmic order, but that in data obtained by most workers, departures are masked by failure to obtain results for the numbers of survivors in the early stage of disinfection because of the rapidity with which this is passed. At low concentrations of phenol the distribution of resistance, measured in terms of survival times, was approximately normal, and symmetrical sigmoid survivor curves were obtained for such populations; however, resistance may frequently be distributed in a slightly skew manner, which might explain the various deviations from the sigmoid curve which have been observed. When exponential curves are analyzed in terms of distribution of resistance, extremely skew curves result. This fact has frequently been cited to refute the fact that resistances may be distributed among the individual bacteria of a test population and to support





mechanistic theories of inactivation of bacteria.

Early work is often difficult to evaluate. Withell (1942) worked with cultures which appear to have been washed off an agar surface and must therefore have been of extremely heterogeneous physiological condition. Jordan and Jacobs (1944 a) worked with a batch culture grown under carefully controlled conditions; however, since dry air was used to maintain the culture volume it appears that disinfection experiments were carried out in the presence of high concentrations of metabolites and metabolic end-products. In Withell's experiments up to 30 sec were allowed in which bacteria and bactericide were mixed, before the first count (at 0 time) was determined. Jordan and Jacobs (1944 a) appear to have allowed 5 min for mixing phenol with the culture before estimates of survivors were made. It is the feeling of the author that abnormal results of many workers can be explained by the different experimental conditions used, but in some cases, where experimental conditions vary markedly, attempts to compare results are futile and meaningless.

A fresh approach to the problem of disinfection survivor curves was taken by Eddy and Hinshelwood (1953) and Eddy (1953 a, b). Cultures of Bacterium lactis aerogenes were grown in a synthetic medium and cultures were washed before exposure to the lethal agent. From the results Eddy (1953 b) suggested that death of a bacterial cell involves not only a progressive destruction of



essential cell activities, but may also be influenced at some stage by a random combination of various independent reactions between the lethal agent and susceptible cell components. The shape of the survivor curve appears to be dependent on the rapidity with which the independent events reach critical levels, resulting in death of the cell. These authors suggested that inherent differences in resistance of individual cells do not cause major variations in survivor curves and further that all types of survivor curves may be explained by this theory because death is caused by destruction of enzyme systems.

#### CRITERIA OF DEATH OF MICRO-ORGANISMS

The most common criterion used to determine whether any single organism is living or dead is to place it in a favorable environment, wait for an arbitrary length of time and then score those organisms which have multiplied as being alive. The prerequisite of multiplication in a limited period of time as criterion of life seems to be unacceptable to most biologists; even to the microbiologist it presents a number of obvious problems. One need not further discuss the question of enumerating dormant bacterial spores without some treatment to encourage germination, or bacteriostatic effects of traces of disinfectant remaining on bacteria removed from contact with a lethal agent; however, in disinfection the recovery and enumeration of survivors is used as an indication of the effectiveness of the agent under test. Davis (1960 b) noted





that selective media tend to be inhibitory even to the organisms for which they are designed and are therefore of no special advantage in disinfectant testing. Media permitting luxuriant growth of the test organisms usually allow the greatest opportunity for recovery of damaged cells. Thus nutrient agar or media containing yeast extract are most frequently used for routine testing. Milbauer and Grossowicz (1959 a) recently showed that chlorine-inactivated E. coli would show greater survival when plated on nutrient agar than on minimal agar. They claimed that the greater survival of bacteria on rich media represented reactivation of chlorine-inactivated cells. Kelner (1949) first demonstrated reactivation of E. coli inactivated by ultraviolet light and subsequently exposed for 45 - 60 min to visible light of wavelength of 5100 Å. Whereas reactivation of bacteria and bacteriophages by visible light appears to have become an accepted fact, the report by Heinmets et al. (1954), that heat and chlorine-inactivated E. coli could be reactivated by being incubated in buffer containing tricarboxylic acid cycle metabolites, resulted in a controversy. Reactivation of inactivated bacteria could only be obtained when the cells were incubated for 24 h at 37°C in buffer fortified with the metabolites before counts were made. No reactivation could be shown when recovery media (agar) were fortified with the metabolites. Garvie (1955) objected to the evidence suggesting that chemical reactivation occurred, and showed that her E. coli strain 555 could multiply in buffer after being exposed to chlorine



and that the growth was enhanced by various Krebs cycle intermediates. Chambers et al. (1957) also doubted that chlorine "killed" bacteria could be reactivated but they were able to "reactivate" bacteria "killed" by chlorine provided some survivors remained after treatment. When only dead cells were left after exposure to the disinfectant, no reactivation was observed on addition of tricarboxylic acid cycle intermediates. The increase in survivors obtained on incubation with metabolites after exposure to low chlorine concentrations was regarded as multiplication rather than reactivation. In a critical investigation, Hurwitz et al. (1957), used the most probable number approach to investigate the occurrence of reactivation. Chlorine and heat treated E. coli were exposed to "reactivating" metabolites on Millipore filters before the filters were transferred on to nutrient medium. They concluded that reactivation did not occur. It is obviously important to know whether bacteria can be reactivated after treatment by disinfectants and heat. The claim of Milbauer and Grossowicz (1959 a) that reactivation of chlorine-treated bacteria was obtained by immediate plating on rich and minimal agar again draws attention to the problem. Two possible conclusions may be made from the studies on reactivation:

1. Certain enzyme loci involved in utilization of energy-yielding carbohydrates, such as glucose, are very susceptible to destruction by chlorine.
2. After destruction of certain enzymes by chlorine, tricarboxylic acids would serve as immediately utilizable energy





producing compounds. This might lead to cell repair or even induction of other enzymes leading in turn as contended by Heinmets et al. (1954) to partial or complete restoration of the enzymic damage caused by the disinfectant.

### CHLORINATION

The outstanding characteristic of chlorine is the ability of minute concentrations to destroy large numbers of bacteria in a short contact time. However, the very property which makes it so active in this respect, the power of interacting with the constituents of the bacterial cell, carries with it the ability to react with a large number of organic and inorganic substances. Chlorination is therefore no simple process. A large number of active molecules of chlorine is required to enter a bacterial cell before it is killed. The efficiency of chlorine as a disinfectant not only depends on concentration, contact period, temperature and pH of the reaction mixture but the presence or absence of organic material or ammonia also drastically influence the chlorination process.

#### Active Compounds of Chlorine

Compressed elemental chlorine gas is probably the commonest form in which chlorine is used for purification of water and for sewage treatment; however, calcium and sodium hypochlorites combined



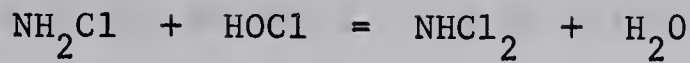
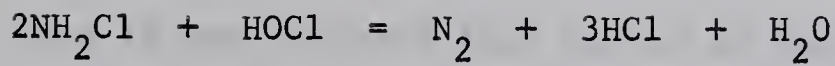
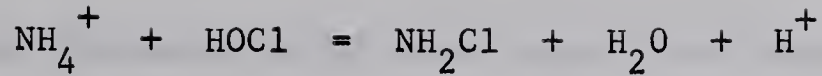


with trisodium phosphate, also liquids containing sodium hypochlorite are widely used for disinfection purposes. The stability of hypochlorite solutions is affected by temperature, concentration and pH. Solutions rendered alkaline to about pH 9.5 often by mixing sodium and calcium hypochlorite are quite stable; however, solutions with high available chlorine content are less stable than weaker ones. Storage of hypochlorites in cool dark places to prevent deterioration is recommended regardless of strength.

Chlorine dioxide appears to be a relatively new chlorine bearing disinfectant. It has been used for treatment of water and it is of special interest since its bactericidal power increases with increasing pH values which is an effect opposite to that recorded for chlorine and other chlorine compounds. This complementary situation would be important from the standpoint of disinfectant practice as well as the further elucidation of mechanisms of chlorine acting on micro-organisms.

When chlorine reacts with ammonia or amino groups in organic nitrogenous substances a number of chloramines are formed. The relative proportion of the substitution products depends on pH and relative concentrations of the reagents when ammonia and chlorine react (Allen and Brooks, 1952) and may be illustrated as follows:





One immediately recognizes these reactions as the phases in the "break point" curve as illustrated in Fig. 1.

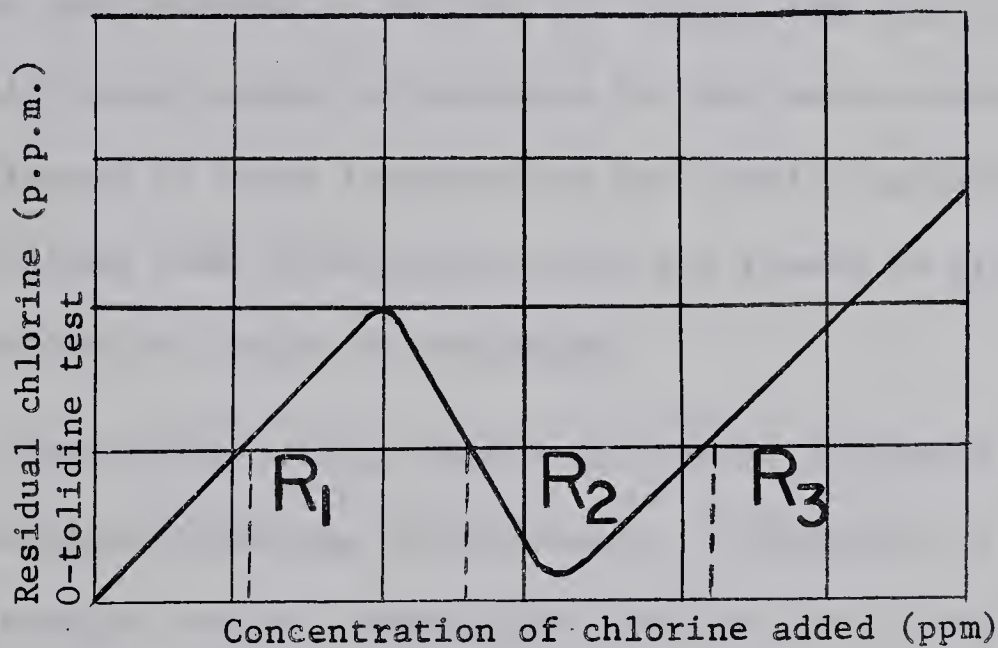


Fig. 1. Typical break-point curve for chlorine and ammonia. Taken from Allen and Brooks (1952).

The initial rise in the curve represents formation of mainly monochloramines. As higher amounts of chlorine are added the chloramines decompose until the break-point is reached. If chlorine is added to react to points beyond the break-point then free chlorine and nitrogen trichloride appear. Since free chlorine is much more active as a disinfectant than chloramines (although both substances give the same color reaction with o-tolidine) it





follows that the bactericidal rate beyond the break-point will be greater. Thus it was observed that spores of Bacillus metiens were killed in 83, 88 and 2.7 min at points  $R_1$ ,  $R_2$  and  $R_3$  when the residual chlorine at these points was found to be 24.9, 24.2 and 22.5 ppm respectively.

Similar curves may be obtained for the interaction of amino acids and chlorine at neutral pH, though the reaction of chlorine with amino groups of proteins is much more complex. The compounds formed by these interactions are usually bactericidal though much less than hypochlorites, and may result in prolonged bactericidal action during chlorination.

There exist a large number of organic compounds which are also chlorine releasing disinfectants. Chloramine-T, halazone and azochloramide are the common names for the best known products. Chloramine-T and azochloramide have a prolonged sustained anti-bacterial activity and have been used for treatment of infected wounds. Halazone was found suitable for disinfecting small amounts of drinking water when dispensed in tablet form; however, generally speaking these and other newer organic chloramines appear not to be used too widely. Frequently their chemical structure is quite complex and it is not certain whether they kill bacteria in the same manner as the inorganic chlorine bearing disinfectants.



## The Effect of Various Chemical Substances on Chlorine

Although chlorine is a very reactive chemical there is in fact a somewhat limited group of compounds which will react with chlorine in dilute solutions at low concentrations, at the temperature and pH ranges encountered during disinfection. However, since these reactions may change the concentration of available chlorine or result in undesirable products from the standpoint of public health or chlorination efficiency, a clear understanding of these reactions is important.

Compounds reacting with chlorine may broadly be divided into two types - those which form germicidal compounds and those which do not. Sulfides, thiosulfates and ferrous salts react quickly with chlorine to destroy bactericidal action. It is for this reason that sodium thiosulfate is commonly used as a "neutralizer" of residual chlorine during disinfectant testing or when bacterial counts are done to assess the bactericidal efficiency of a water treatment process.

Whereas the reaction between sodium thiosulfate results in non-bactericidal non-toxic substances, chlorine may react with thiocyanate to form cyanogen chloride. This substance is a slow acting persistent bactericide and is quite stable in the presence of organic matter; it is highly toxic to man and animals and also to fish. Thiocyanate which may be discharged into sewage works is commonly oxidized by bacterial action to ammonium sulfate





prior to chlorination of the sewage.

Allen and Brooks (1952) noted from the literature and their own work that non-nitrogenous organic compounds reacted only slightly with chlorine. Thus solutions of glucose, maltose, lactose, sucrose, raffinose, cholesterol, sodium oleate, sodium palmitate, glycerol, starch, acetaldehyde, methyl alcohol and ethyl alcohol created no appreciable chlorine demand.

As pointed out in the previous section, chlorine reacts with organic and inorganic nitrogenous compounds to form bactericidal chloramines. However, reaction with aromatic or heterocyclic amino acids, that is partial oxidation or chlorination of the ring, may result in non-bactericidal compounds.

Chlorine, like many other substances, may apparently be adsorbed by inert suspended matter, such as agar and powdered charcoal and lose some or all of its bactericidal power. The presence and use of the latter compounds in some water treatment processes may lead to problems since the adsorbed chlorine may still be detectable by the o-tolidine test.

How may the reactivity of chlorine with other substances influence chlorination tests?

1. Chlorine may react chemically with organic and inorganic compounds to give rise to non-bactericidal compounds. Reaction with a competitive substance e.g. sodium thiosulfate may take place more readily than the combination with bacterial protein.





2. Chlorine may form an insoluble compound with organic matter. In some cases it has been suggested that such denatured proteins are deposited on bacterial cells and form a protective coating against the lethal agent.

3. Particulate matter such as charcoal may adsorb chlorine and render it unavailable.

4. Naturally occurring substances such as serum, milk and even excreted metabolic end-products from the cells may interact to form non-bactericidal compounds or compounds which act slower on bacteria such as chloramines.

The sustained, slower bactericidal action of hypochlorites after free available chlorine disappeared in the presence of milk was attributed to chloramine formation by Labots and Galesloot (1964). The same authors (1963) showed that chloramine formation on the addition of culture media had little effect on bacterial survival in short exposure time tests; however, the action of the chloramines formed could become pronounced on long time exposure. Friberg (1957) illustrated the formation of similar amounts of combined available chlorine on exposure of live and heat-killed bacteria to small amounts of free available chlorine.

It is obvious that any test procedure for the evaluation of chlorine type disinfectants, which does not account for chemical interferences illustrated above, will be inadequate in forming guide rules for the use of such disinfectants in practice; however,



it is just as obvious that these interfering reactions must be largely eliminated in experiments to determine how chlorine inflicts death on bacteria.

### The Effect of Temperature

When the effect of temperature on bactericidal activities of chlorine compounds is studied, it is found that the efficiency of these bactericides increases with temperature. It is important therefore that determinations of temperature coefficients should be carried out in ranges where temperature alone does not contribute to the lethal effect. Brazis et al. (1958) showed that three to four times as much free available chlorine was required to produce a 99.99% sporicidal effect at 4°C as at 22°C when other factors remained constant. They also noted that the same relationship existed when chlorine was expressed as hypochlorous acid.

Butterfield et al. (1943) showed that vegetative bacteria are also affected by increases in temperature. Chloramine is more affected in its bactericidal efficiency by increases in temperature than chlorine; however, the temperature effect of both types of disinfectants was more marked throughout the chlorine concentrations studied at alkaline than at neutral pH. The highest temperature used in these experiments was 25°C, eliminating heat kill as an explanation.





### The Effect of Concentration

As the concentration of a lethal agent increases, one can generally expect to note some increase in the rate of death produced by the agent on a bacterial population. The effect of concentration of disinfectants on bacteria has been expressed by the formula:

$$n \log C = \log k - \log t$$

where C is the concentration of disinfectant, t is the time required to destroy a given portion of the bacteria, and n is the concentration exponent. If log t is plotted against log C, the slope of the line gives the concentration exponent, n. When the above equation is written as  $C^n t = K$ , one can see that the concentration exponent will have a direct effect on the exposure time. Thus mercuric chloride, for which n is 1, will require twice the time to cause a certain degree of damage when the concentration is halved, whereas phenol, for which n is 6, will require a 64 times increase in time to achieve the same damage.

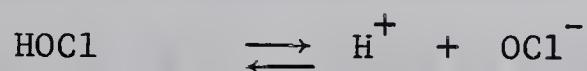
With respect to concentration, chlorine and chloramines behave much like mercuric chloride. Allen (1950) calculated from data published by other workers that the concentration coefficient of hypochlorite and simple chloramines may be between 0.5 to 1.5 depending on temperature and pH. Chlorine is therefore a disinfectant which is not disproportionately weakened by dilution;



however, in view of the minor effect of the concentration exponent as compared to the influence of pH on available chlorine one can frequently disregard this contribution.

### The Effect of pH and the Mechanism of Disinfection

The disinfectant power of chlorine, whether in the form of hypochlorite, simple chloramines or chloramine-T is greatly affected by changes in pH value. So important is this effect that in most discussions the influence of pH is in fact treated as an integral part of the mechanism of chlorination. It appears that the pH value may be correlated with the concentration of undissociated molecules of hypochlorous acid. A dilute chlorine solution is hydrolyzed to form hypochlorous acid, which further dissociates into its constituent ions:



It has been shown that the sporicidal, bactericidal and cysticidal efficiency of chlorine depends on the amount of undissociated hypochlorous acid in the system. Since this dissociation is largely affected by pH, Allen (1950) compiled a table showing the percentage undissociated hypochlorous acid in a system as pH varies:



| <u>pH value</u> | <u>Percentage chlorine as HOCl</u> |
|-----------------|------------------------------------|
| 10              | .03                                |
| 9.0             | 3.0                                |
| 8.0             | 21                                 |
| 7.43            | 50                                 |
| 7.0             | 73                                 |
| 6.5             | 91                                 |
| 6.0             | 96                                 |
| 5.0             | 99.7                               |
| 4.0             | 99.6                               |
| 3.5             | 98.6                               |
| 3.0             | 95.7                               |
| 2.5             | 87.7                               |
| 2.0             | 69.0                               |
| 1.5             | 41.5                               |
| 1.0             | 18.2                               |

The concentration of  $\text{Cl}_2$  is appreciable only at pH values below 5.0 and that of  $\text{OCl}^-$  ions only at higher pH values.

Butterfield et al. (1943, 1946) studied the influence of pH on both chlorine and chloramine disinfection of coliforms and enteric pathogens. It can be concluded from these experiments that free hypochlorous acid is directly involved in killing bacteria. Thus the efficiency of disinfection generally decreases as the pH varies from about 7.0 to 9.5. There is evidence that exponential time survivor curves at pH 6.0 can be converted to sigmoid curves as the pH is changed to 9.5 and other factors remain constant. It is obvious that the largest contribution in this conversion of time survivor curves is the progressively lower amount of hypochlorous acid in the system as the pH increases; however, one should not dismiss drug-resistance because of internal pH changes as described by Good (1954). For example Wattie and Butterfield (1944) concluded





that cells of Eberthella typhosa were more sensitive to free chlorine with increasing pH, because of some function of their capsular substance and suggested that this might be less permeable to chlorine at lower pH values. Experiments on the interaction of bacteria and chlorine below pH 5.0 appear to be lacking; however, the hypochlorite  $\text{OCl}^-$  ion appears to possess low penetrating power, and a few claims of renewed increases in bactericidal efficiency at the pH values above 10.0 can probably be dismissed as a result of the alkaline environment directly on the bacteria, rather than of disinfection action. Extensive death caused by acid conditions alone (pH 2.0 to 3.5) was illustrated for Bact. lactis aerogenes by Eddy and Hinshelwood (1953), but it was not stated what type of acid was used.

It can probably be concluded that hypochlorous acid is the active bactericidal compound in chlorine disinfection. Chlorine is regarded as largely bactericidal, though there exists at least one paper attributing bacteriostatic action to chlorine (Mudge and Smith 1935); however, Knox et al. (1948) concluded that oxidation of sulfhydryl groups of essential enzymes would serve to explain the rapid bactericidal action of chlorine. Barron (1951) supported the importance of sulfhydryl groups as reactive centers with lethal agents. Friberg (1957) noted that combined available chlorine substances (organic chloramines) are even formed when washed E. coli cells are treated with chlorine, and he



suggested that this chloramine formation may be a part of the lethal process. He suggested that the bacterial chloramines may result in prolonged lethal action.

Further evidence of enzymic involvement in chlorine-resistance is given by Farkas-Himsley (1964) and Milbauer and Grossowicz (1959 a, b). These papers suggest carbohydrate utilizing enzymes are most directly involved because:

1. The glucose utilizing enzyme system appears to be inactivated most rapidly as shown by reactivation experiments.
2. Chlorine-susceptible, minimal agar grown E. coli would not recover chlorine-resistance when the minimal agar was fortified with proteinaceous materials such as Bovril or peptone; however, when the minimal agar was fortified with yeast extract, which may consist of up to 40% carbohydrates, the bacteria were almost as resistant as those grown on nutrient agar.

Chlorine is a good example of the reasons of the paucity of knowledge concerning the processes involved in disinfection. The chemistry of chlorine compounds is generally well known, and the reaction which chlorine undergoes with other inorganic and simple organic compounds has been extensively studied, however, frequently results from such simple experiments are used to make deductions of what happens when chlorine and bacteria interact.





## CONTINUOUS CULTURE

The need for more carefully grown bacterial cultures used for inocula in disinfectant testing was emphasized by Jordan and Jacobs (1944 a), who designed their experiments so that phenol could be added directly to a steady-state culture. This, of course, resulted in complications because the germicide had to act in the presence of nutrient medium, and that the whole bacterial culture was exposed to the disinfectant allowing no bacteria in the steady-state from the same culture for repeat experiments. The author felt that information could be gained on the causes of variation in disinfectant resistance of bacteria if a steady-state bacterial culture could be maintained over prolonged periods of time, which would permit aliquots of such a culture to be exposed to the lethal agent. This would allow the assessment of changes in the disinfection process caused by variation in the bacterial culture rather than by variation of lethal conditions.

This approach appeared to be possible because of the invention of continuous culture of micro-organisms. In the United States Novick and Szilard (1950) were the first to present an apparatus design and theoretical and mathematical reasons to make possible the use of continuous culture in laboratory situations; however, so rapid has been the development and so great has been the interest that the subject was reviewed twice in less than ten years (Novick, 1955 and James, 1961).



### Design of Apparatus and Theory of Operation

Continuous culture equipment is designed to provide growing micro-organisms with unchanging environment throughout the experimental period. There are two types of instruments which allow automatic addition of nutrient and attainment of a steady state at a relatively uniform population density or growth rate. In the turbidostat, nutrient is added in response to increased turbidity of the culture, thus maintaining a uniform density through dilution and wash-out of excess cells. The turbidostat consists of a temperature-controlled growth vessel linked to a photometer. When the bacterial concentration reduces the percent transmittance to a preset level, a pump is activated which allows a pre-determined amount of medium to be introduced into the system. Growth of bacteria on the walls of the culture vessels has created some problems in this type of equipment, however various types of windshield wipers have been used to reduce this effect. It is obvious that the growth curve will not strictly be a straight line once the steady state has been obtained; however, turbidostats are very useful for cultivating cells which have a low growth rate or complex or unknown growth requirements.

A simpler and apparently more frequently used device is the chemostat which has the desirable property of being self-regulating. In this apparatus the bacterial growth is kept at a constant level by a growth-limiting factor, the fixed rate of input





of culture medium. Henderson et al. (1958) wrote:

"If the flow of medium is set to a suitable value and then held constant, the system automatically adjusts itself to a steady state, in which the concentrations of bacteria and of growth substrates in the culture vessel, and all other variables such as cell respiration, etc. remain constant indefinitely, so long as the dilution rate remains unaltered. Those who have never used such an apparatus may be surprised to hear that bacteria will behave in so accommodating a manner. ....It is found in practice that the steady-state operation is possible over a wide range of dilution rates. There is always an upper limit or critical dilution, above which wash out occurs, i.e. the bacteria are washed out of the growth vessel faster than they can grow, so that their concentration falls to zero. There is a less well defined lower limit at dilution rates around  $0.03 \text{ hr}^{-1}$ , at lower dilution rates the cells appear to go into a kind of lag state and cease to grow. Between these limits, steady-state operation is possible at any desired dilution rate."

The above quotation indicates that simple operation of continuous culture, such as used in the present experiments, does not require involvement in the complicated mathematics which have





been published for population studies with chemostats; however, the dilution rate may be found in the following manner. In the chemostat the mean residence time of a particle in the culture vessel is determined by the ratio of flow rate (f) to culture volume (v). This is defined as the dilution rate D:

$$D = \frac{f}{v} \text{ hours}^{-1}$$

The dilution rate gives the number of volumes of medium passed through the growth vessel in one hour and its reciprocal  $1/D$  is the mean residence time.

It is easily seen that when a continuous culture is in a steady-state, the exponential growth rate of the cells must be equal to the dilution rate. The continuous culture apparatus allows control of the growth rate by appropriately setting the dilution rate. In the chemostat the growth-limiting substrate in the culture medium controls the growth rate of the organisms. This is possible because once the steady-state concentration of bacteria is reached, the growth-limiting factor is used up at the same rate as it is added. The bacterial culture can be maintained in a chemostat without changing its physiological condition as long as the dilution rate is carefully regulated. It is obvious that this should allow for harvesting of reproducible inocula for disinfection experiments while the chemostat is maintained in a steady-state.



## MATERIALS AND METHODS

### PREPARATION AND COMPOSITION OF MEDIA, SUBSTRATES AND OTHER SOLUTIONS

#### Bacteriological Media

With the exception of eosin methylene blue agar (EMB) which was obtained from the Baltimore Biological Laboratory, Inc., the media used in this investigation were those described by Lederberg (1950) and had the following composition.

| Minimal Medium<br>(g/l)                         |     | Complete Medium<br>(g/l)        |      |
|-------------------------------------------------|-----|---------------------------------|------|
| Glucose                                         | 1.0 | Casein digest*                  | 10.0 |
| K <sub>2</sub> HPO <sub>4</sub>                 | 7.0 | Yeast extract (Difco)           | 5.0  |
| KH <sub>2</sub> PO <sub>4</sub>                 | 2.0 | K <sub>2</sub> HPO <sub>4</sub> | 3.0  |
| Na <sub>3</sub> citrate . 5H <sub>2</sub> O     | 0.5 | KH <sub>2</sub> PO <sub>4</sub> | 1.0  |
| MgSO <sub>4</sub> . 7H <sub>2</sub> O           | 0.1 | Glucose                         | 5.0  |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 1.0 |                                 |      |

\*casein hydrolysate, enzymatic-Nutritional Biochemical Corporation

Minimal broth. This was prepared by dissolving the glucose in 100 ml distilled water, and the other ingredients in 900 ml water, and was dispensed separately in 1 ml and 9 ml quantities respectively. One ml of 1% sterile glucose solution was added aseptically to the buffered portion of the minimal broth just before inoculation. Minimal broth prepared by autoclaving glucose separately from the other ingredients and mixing as above gave a clear solution; if all the ingredients





were autoclaved together degradation and interaction of glucose with other ingredients resulted in a yellow medium. This was of great importance when long heat treatments had to be made. With the sterilization of 18 l lots of broth required for operating the chemostat, a correspondingly longer "pre-heating" period was required in the autoclave to allow the medium to attain sterilizing temperature. Under these conditions the separate sterilization of glucose solution from the rest of the medium was of even greater importance.

Complete medium. This was prepared by adding all the ingredients to the distilled water and autoclaving for 15 min at 121°C. However, when an 18 l lot of complete medium was prepared for use in the chemostat, only 1g/l of glucose was added. In this case the glucose solution was sterilized separately in the glass bottle of the medium reservoir as described below.

Solid media. Solid media were made by adding agar (Difco) at a concentration of 1.5% (w/v). Complete agar and EMB agar were made-up in 1 or 3 l amounts in hot water. The agar was dispensed into 300 ml flasks with ground glass necks for autoclaving. The molten agar was poured into Petri dishes in 10 ml amounts with the aid of a tilt dispenser. Agar plates required for making spot-plate counts were dried overnight in the 37°C incubator and then stored in a 6 to 10°C refrigerator for a maximum of 2 weeks. Plates used for making spot-plate counts were removed from the refrigerator



2 to 3 hours before use and stored at room temperature to assure rapid drying of the plates after inoculation.

Buffered diluent. To prepare buffered diluent, 1.25 ml of stock phosphate buffer was added to 1 l distilled water. Bacto-peptone at a concentration of 0.05% was added to the buffered diluent and the peptone water was dispensed in 99 ml and 9 ml amounts in screw-cap glass containers for autoclaving. The use of 0.05% peptone in dilution blanks caused less foaming than the 0.1% which is commonly used.

The 0.25M stock phosphate buffer was prepared according to the method of the American Public Health Association (1960 a). Thirty-four g of  $\text{KH}_2\text{PO}_4$  was dissolved in 500 ml of distilled water and adjusted to pH 7.2 with N NaOH. The resulting 0.25M  $\text{KH}_2\text{PO}_4$  buffer was autoclaved in convenient quantities.

If residual chlorine had to be neutralized, the 0.05% peptone water was fortified with 1% sodium thiosulfate and dispensed in 9 ml quantities. Neutralizer was used only to make the first decimal dilution after bacteria were removed from chlorine.

#### Substrates and solutions for the tetrazolium tests.

As described in the section on tetrazolium salts, these substances can be reduced to formazan by most dehydrogenase systems. It is therefore possible to use a tetrazolium test to estimate relative metabolic damage (dehydrogenase damage) inflicted on a bacterial





population by a lethal agent. Since dehydrogenases occur at various places in the sequences of metabolic pathways responsible for utilization of carbohydrates, a number of substrates such as casein digest, yeast extract, glucose, sodium lactate, malic acid and complete broth were used singly or in various combinations to estimate such damage. The type of substrate used is indicated in the appropriate experiments.

To perform the tetrazolium test various other solutions used as indicators, neutralizers or blanks (to equalize volumes) were required. Following is a list of the composition and preparation of all substrates and solutions used:

- a) Casein digest - 24 g per 100 ml of 0.25M  $\text{KH}_2\text{PO}_4$  buffer
- b) Yeast extract - 12 g per 100 ml of 0.25M  $\text{KH}_2\text{PO}_4$  buffer
- c) Glucose - 12 g per 100 ml of distilled water
- d) Neutralizer solution - 1 or 2% sodium thiosulfate made up in 0.25M  $\text{KH}_2\text{PO}_4$  buffer
- e) 2, 3, 5 triphenyl - tetrazolium chloride (TTC) solution (The British Drug Houses Ltd.) - 1000 to 10,000 ppm solutions were made up in 0.25M  $\text{KH}_2\text{PO}_4$  buffer and used as required to give the final desired concentration
- f) Solution blanks - consisted of distilled water or 0.25  $\text{KH}_2\text{PO}_4$  buffer as required by the composition of the substrate solution mixture
- g) Sodium lactate - 1 M solution in distilled water





- h) Glucose - 1 M solution in distilled water
- i) Malic acid - 1 M solution in distilled water  
(adjusted to pH 7.0 with NaOH)
- j) Complete broth - was used as the substrate in early experiments to establish the tetrazolium staining pattern of E. coli.

Substrates a - c were warmed to 32°C and vigorously stirred with a magnetic stirrer. They were filtered under vacuum through sintered glass followed by a Whatman no. 1 filter in a Millipore filter holder. The solutions were dispensed in 5 ml quantities in screw-cap test tubes and sterilized at 121°C for 15 min. The casein digest solution alone did not result in a great deal of formazan formation, as would be expected, and was not used extensively.

Substrates d to j were usually dispensed in 10 ml quantities in screw-cap test tubes and sterilized as the other substrates. The TTC solutions were cooled rapidly after sterilization and could be kept in the dark at room temperature for months without any apparent formation of formazan. When such solutions containing 4800 ppm were kept on the top of the bench exposed to artificial light in the room only the faintest pink was noticed after prolonged exposure (>4 h).



### Stock solutions of chlorine and chloramine-T

A solution of commercial sodium hypochlorite containing approximately 12% available chlorine was diluted with demineralized, distilled water in a brown glass bottle to give a 1000 ppm concentration of available chlorine as determined by the standard thiosulfate titration (American Public Health Association, 1960 b). The solution was quite stable when stored at 6 - 10°C.

A chloramine-T solution containing 500 ppm available chlorine was also prepared in the same manner. These solutions were checked for available chlorine before use and adjusted if necessary.

### Growth and Maintenance of Bacteria

Maintenance of stock cultures. A freeze-dried culture of Escherichia coli 555 was obtained from the National Collection of Dairy Organisms, University of Reading, England. This organism was selected for study since it is frequently used as a test organism for evaluating chemical disinfectants. The original growth from the freeze-dried culture was obtained by inoculating the contents of the vial on to a complete agar slope. After 24 h at 32°C subcultures were made on to agar slopes and incubated 32°C until growth was clearly evident. Such stock cultures were stored at about 6°C in a walk-in cooler and were subcultured at intervals of about 3 months. E. coli 555 was used for all work with continuous culture; however, a capsulated strain of Aerobacter aerogenes was used for





a limited amount of work. This strain grew well on the media described above for culturing E. coli and was maintained in the same manner.

Preparation of inocula and batch culture. Stock culture slopes were transferred from the cooler and incubated at 32°C for 24 h before being subcultured into minimal broth and incubated for a further 24 h. At least three transfers in minimal broth were made before a culture was used to inoculate the chemostat or a batch culture to be used for harvesting organisms for disinfectant studies. Active growth of cultures was maintained by repeated 24 h subculture into minimal broth. New starts from stock cultures were made frequently.

Batch cultures of bacteria were made by inoculating 500 ml of broth in 1 l Erlenmeyer flasks with 1 ml of an actively growing culture in minimal broth. Aeration of batch cultures was achieved either by stirring the broth with a magnetic stirrer or by air previously filtered through a cotton wool filter described later. Batch cultures were incubated in a water bath at 32°C for 24 h or as specified.

#### Continuous Culture of E. coli

Description of apparatus used. Since commercially available equipment at the time this work was started was limited, it became necessary to design a chemostat to suit our own purposes. The objectives in the design were:



1. Economy, which demanded the use of equipment normally available in the laboratory.
2. Simplicity of operation in order to assemble the whole apparatus before sterilization, and yet to allow for a number of simple connections for changing media and product reservoirs.

The apparatus which was designed is shown in Fig. 2. The culture vessel (A) consisted of a water jacketed Bellco spinner flask, with 1000 ml capacity to the overflow (a), altered as required. Slyter et al. (1964) reported the use of such a flask in a continuous culture device for rumen micro-organisms at the time this apparatus had been developed. The magnetic stirring device (b) of the flask was provided with an air distributor disc (c) cut from a 3/16 inch Teflon sheet. A similar disc could be mounted on the glass stirrer at position (d) just above the liquid level to aid in destruction of foam if this presented a problem. The inoculating port (e) consisted of a rubber seal inside the port and was covered by a ground glass dome.

The feeder-head (B) was adapted from the device designed by Jordan and Jacobs (1944 b) and allowed the introduction of air (f) and medium (g) at the same place to the bottom of the vessel to facilitate mixing. It also prevented contamination of the medium jet (h) by the bacterial aerosol, (which caused back-growth into medium reservoir in the first apparatus assembled in this laboratory).

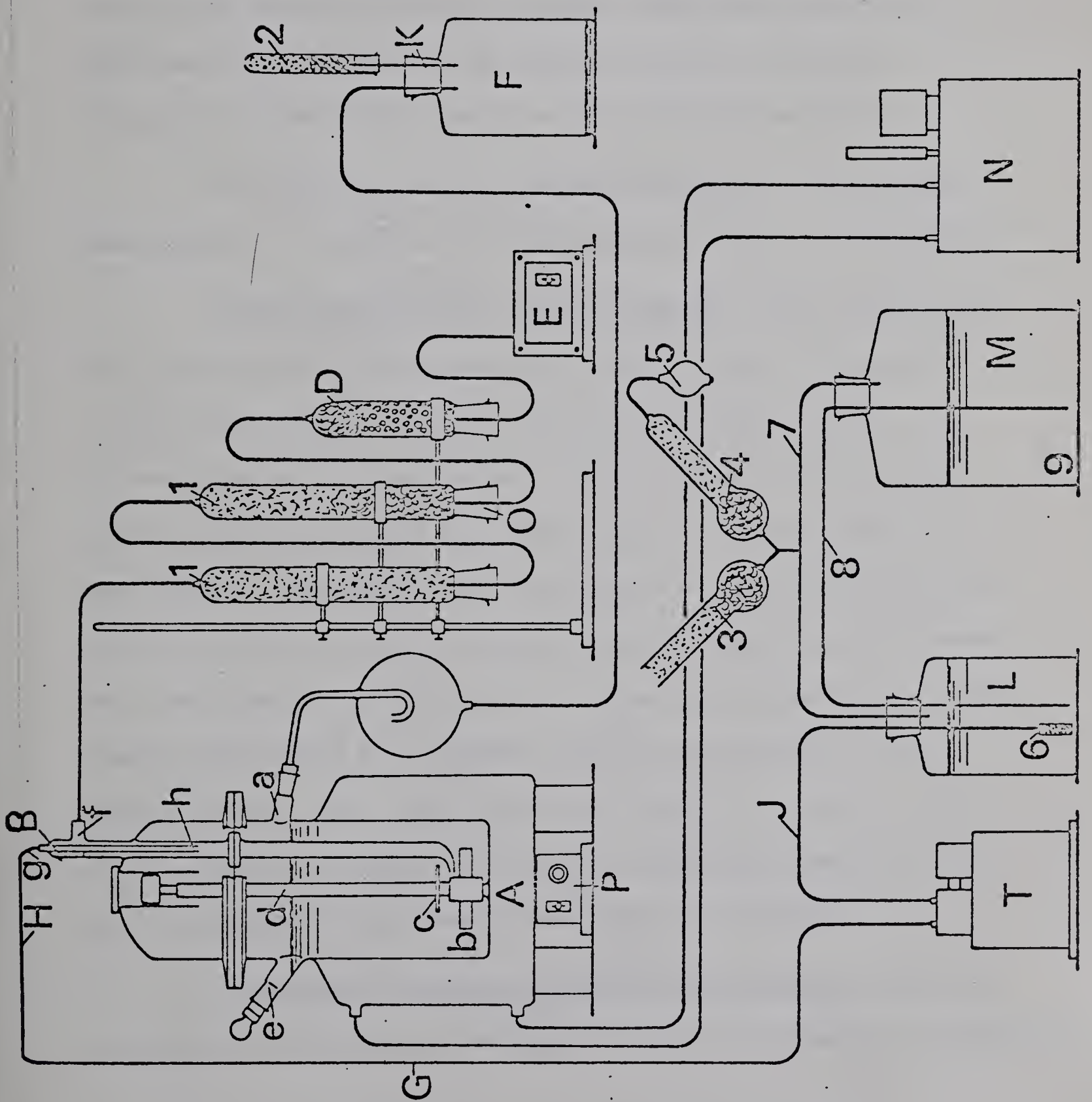


Fig. 2. CHEMOSTAT FOR CONTINUOUS MAINTENANCE OF BACTERIAL POPULATION

- A. Culture vessel
  - a. constant volume overflow and gas escape
  - b. magnetic stirring device
  - c. and d. Teflon discs for distribution of air and destruction of foam
  - e. inoculating and sampling port
- B. Feederhead
  - f. air inlet
  - g. medium inlet
  - h. medium jet (flow could be calibrated by counting drops of medium per minute)
- C. Airfilters
  - 1. main airfilters
  - 2. product reservoir airfilter
  - 3. sterilization exhaust filter
  - 4. pressure equalization filter
  - 5. rubber bulb (used to start siphon only)
- D. Air dryer containing  $\text{CaCl}_2$
- E. Air supply and flow measuring device
- F. Product reservoir
- G. Tygon medium supply line with hypodermic needles
- H. Connection-consisting of observation tube with Luer-Lok and male ground glass joint.
- J. Connection consisting of observation tube with Luer-Lok to rubber tubing.
- T. Peristaltic pump (Sigmamotor Model T-8)
- K. Product reservoir closure
- L. First stage of medium reservoir (1 gallon glass bottle)
  - 6. sintered glass filter on end of medium supply line
- M. Second stage of medium reservoir (5 gallon polypropylene bottle)
  - 7. air exchange line
  - 8. siphon
  - 9. magnetic stirring bar
- N. Circulating water bath
- O. Air line break for sterilization
- P. Magnetic stirrer









The air, after passing through  $\text{CaCl}_2$  (D), was filtered through Pyrex glass wool, glycerine-treated cotton and cotton wool in filters (I). The medium supply line (G) consisted of Tygon tubing ending in blunt 3-inch hypodermic syringe needles inserted into the tubing at points H and J. The line could be detached at these points easily because observation tubes with Luer-Loks were connected to the medium reservoir by rubber tubing and to the feeder-head by a 5/20 ground glass joint.

The two stage medium reservoir consisted of a 1-gallon glass bottle (L) connected to a 5-gallon polypropylene bottle (M).

Sterilization of the culture assembly. For sterilization the culture assembly was mounted on a metal stand. The airfilter at (O), the hypodermic needle at (J) and the rubber stopper at (K) were wrapped in aluminum foil. Ground glass joints and the culture vessel just above the liquid line were coated with silicone grease. The assembly was then placed in a horizontal position in the autoclave. After 10 min with free-flowing steam the assembly was sterilized for 20 min at  $121^\circ\text{C}$ . After sterilization the culture assembly was raised to a vertical position and mounted on the magnetic stirrer (P). When the rubber stopper from the air filter at (O), the rubber stopper at K, and the hypodermic needle at (J) had been inserted, the chemostat was ready for operation.

Preparation of medium supply for the chemostat. The two stage medium reservoir was designed to allow sterilization of ingredients





of the medium in different bottles and aseptic mixing after sterilization and cooling. Bottles L and M were connected by a siphon (8) and an air line (7) and a batch of 18 l of medium was prepared at one time. Three l of water was placed into bottle (L) and 15 l into bottle (M). Glucose for 18 l of medium was added to bottle (L) whereas the other ingredients were added to bottle (M). The siphon line was clamped off to prevent mixing during sterilization and filter (4) was clamped at both ends. The medium supply line was clamped off at (J) and the observation tube was covered with aluminum foil. It could be placed into an ordinary steam-sterilizer when bottle (M) was slanted at about 45° and bottle (L) fitted in front. Sterilization consisted of 45 min free-flowing steam and 45 min at 121°C. The prolonged heat treatment was necessary to allow for equalization of pressures and heating of the large volume of liquid involved as mentioned previously. The bottles had to be removed as soon as the pressure of the steam-sterilizer had reached zero to prevent formation of a vacuum and excessive boiling inside the bottles. On removal from the sterilizer, filter (3) was left open until the pressure inside the bottle had equalized. When cooling of the medium started filter (4), which was dry, was opened at both ends. Since the polypropylene bottle (M) had a tendency to collapse, a belt was tightened around its middle after removal from the sterilizer. It may be noted here that stoppers of bottles (L) and (M) were securely bolted down with specially designed clamps, and the tops of the bottles



were tightly covered with foil to prevent dust. After the medium had cooled, it could be mixed by starting the siphon with the help of a rubber bulb (5) and raising bottle (L). When the minimal medium was thoroughly mixed the medium was crystal clear indicating no apparent degradation of ingredients.

Inoculation of the chemostat. After sterilization and assembly, all pumps were started on the chemostat. When the culture vessel had filled a sample was withdrawn from the inoculating port with a syringe. Sterility, optical density and pH were tested and the vessel was inoculated with 5 ml of a minimal broth culture of E. coli. This gave an initial concentration of about  $10^6$  bacteria/ml. The chemostat was normally operated at a dilution rate of .1/h at 32°C. The steady state concentration was reached after about 24 h of operation. Samples to test the operation of the vessel were removed by syringe through the sampling port. Dawson (1963) pointed out that 5% of the volume could be removed without disturbing the steady state and 50 ml was therefore withdrawn to test the operation of the chemostat.

Samples for washing and disinfectant testing were removed either through the sampling port (when sufficient time was allowed to re-establish the steady state before the next sample was taken) or, if a larger quantity was required a sample was collected in the product reservoir (F) which was kept at 0°C in ice water for 2 - 4 h.





Testing operation of the chemostat. The temperature was controlled by circulating water at  $32 \pm 0.1^\circ\text{C}$  from a refrigerated waterbath through the jacket of the growth vessel. The pH was checked daily and total viable bacteria were estimated by the spot-plate method (described later). Turbidity was read on the Klett-Summerson photoelectric colorimeter and corrections were made for the turbidity of the broth by using appropriate blanks. Since in a steady state culture numbers of organisms and metabolic activity should be fixed, various media were used for 20 min checks on tetrazolium reducing ability of 10 ml of the culture at  $32^\circ\text{C}$  as described later. The purity of the culture was frequently checked by Gram-staining and subculture on EMB agar slopes.

The decrease or exhaustion of glucose in the medium flowing from the growth vessel in early runs was frequently tested by the colorimetric determination of glucose by using the alkaline reduction of TTC as described by Mattson and Jensen (1950). After centrifuging a sample collected from the overflow, 10 ml of the supernatant was mixed with 10 ml of N NaOH and the mixture allowed to equilibrate  $60 \pm 25^\circ\text{C}$  in a waterbath. After 6 min 2 ml of a 0.5% ml TTC solution was added to the sample and incubated for 30 min. At this time formazan formation was recorded as glucose present. If no formazan formation was observed it was concluded that glucose had been exhausted.

This method was later abandoned because it was frequently observed that the method resulted in a negative test when incubation of 10 ml of culture with 200 ppm TTC for 20 min gave a positive reduction test. Since this phenomenon was frequently observed when





lack of aeration had resulted in a depression in pH, which was probably due to accumulation of lactic acid during low oxygen tension, it was considered that a negative result using the alkaline TTC reduction test of Mattson & Jensen measured only reducing sugars.

Since the biological oxidation of TTC at pH 7.2 would give a positive response to other utilizable carbohydrates such as lactic acid, this test was preferred as a measure of carbohydrate exhaustion. TTC tests with water were made on every sample taken from the chemostat. A negative test indicated exhaustion of carbohydrate.

#### The Preparation of Washed Bacterial Suspensions for Disinfectant

##### Experiments

Bacteria used for disinfectant experiments were always harvested from liquid media. This was because it had been found early in this work that small colonies of E. coli grown on complete agar sprayed with a 4800 ppm buffered tetrazolium solution stained quickly and evenly, but with giant colonies on complete agar similarly treated, only the periphery of the colony stained rapidly. This suggested that organisms in such a colony may vary greatly in their metabolic activity, probably because of variation in the concentration of available metabolites, and might therefore be quite heterogeneous in disinfectant resistance. The addition of



glucose to the tetrazolium solution did not appear to counteract this effect completely.

#### Washing of bacteria

Centrifuge tubes were filled to contain approximately 40 ml of bacterial broth. After balancing, they were centrifuged in a Servall superspeed centrifuge, with a type SS - 34 rotor, for 10 min at 9000 rpm at room temperature. The supernatant liquid was decanted and the bacterial sediment of each of 4 tubes was washed in 30 ml of buffered dilution water by agitation with a Vortex Jr. mixer.

The tubes were balanced and again centrifuged for 10 min at 9000 rpm. The supernatant was decanted and the bacterial sediment from each of 4 tubes was washed in 10 ml of buffered dilution water. (More dilution water was used if there was a large amount of bacterial sediment). After vigorous stirring on the Vortex Jr. mixer, the suspension was adjusted to an approximate optical density of 1.200 (scale reading of 600) with a Klett-Summerson photoelectric colorimeter. Buffered diluent was used as the blank and a no. 42 filter was used in the colorimeter unless otherwise specified. The bacterial suspension was now filtered through a sterile no. 1 Whatman filter with a diameter of 4.25 cm. The filter had been sterilized in place in a Millipore glass filterholder which was closed by a rubber stopper with a glass wool filter to reduce contamination. The optical density of the bacterial suspension







was not adjusted prior to filtration when the turbidity of the suspension appeared low; however, such adjustment was necessary with heavy suspensions to prevent clogging of the filter. Some cultures of the E. coli strain when grown in complete broth, or the capsulated A. aerogenes used for some experiments, proved difficult to filter through a Whatman no. 1 filter paper. Prefiltration of such cultures through the sintered glass of a sterile, completely closed Millipore filter removed large bacterial clumps which were difficult to break-up and improved the filterability of such cultures.

#### Adjustment of bacterial concentration (optical density)

The bacterial concentration of washed suspensions prepared for disinfectant experiments was adjusted with buffered dilution water to an optical density of 1.040 (scale reading of 520) on the Klett-Summerson photoelectric colorimeter using a no. 42 filter. Buffered diluent was used in the blank.

The Klett-Summerson instrument has a scale graduated in units which are proportional to optical density and the actual numerical values represent the optical density divided by two and multiplied by 1000 (omitting the decimal point). The relation between scale reading R and optical density D is given by Hawk et al. (1954) in the following formula: 
$$\frac{1000 \times D}{2} = R$$

This formula can be changed to:

$$D = R \times 0.002$$



Thus all optical density values in this paper have been obtained by multiplying the Klett-Summerson scale reading by 0.002.

A bacterial suspension with an optical density of 1.040 was found to contain approximately  $10^{10}$  viable E. coli cells/ml. Washed suspensions were stored at 0°C in ice water for up to 2 h and were tempered in a waterbath at the test temperature before being inoculated into the disinfectant solution. In most experiments it was possible to prepare the disinfectant solutions while the bacteria were being centrifuged. Adjustment of the optical density with diluent at the test temperature allowed for exposure of the bacteria to the disinfectant almost immediately.

During the initial exploratory work of the tetrazolium staining phenomenon, the optical density of washed suspensions was determined only to see if bacterial suspensions contained more than  $10^7$  cells/ml. No attempt was made to standardize bacterial concentration of different experiments.

#### Determination of numbers of viable bacteria (spot-plate technique)

Viable bacteria in washed suspensions were determined by the spot-plate technique described in detail by Gaudy et al. (1953). Agar plates prepared and dried as noted above were divided into three equal areas by marking the bottom of each Petri dish. This provided three plating areas per dish. Decimal dilutions of bacterial suspensions were prepared in screw-cap bottles in the conventional





manner. A 0.1 ml pipet graduated in 0.01 ml divisions, was used to place five spots of 0.02 ml from each dilution on the agar surface. A single pipet was used for plating all dilutions of each sample, starting from the highest dilution. The pipet was rinsed out twice with the dilution to be transferred. If plates were sufficiently dried before use, a 0.02 ml volume would spread to a spot with a diameter of 15 mm or less, and would be absorbed by the agar in 10 to 15 min. Plates were incubated at 32°C for 24 h and then counted on the Quebec colony counter. Incubation times in excess of 24 h did not appear to result in increased colony counts. The summation of colony counts from the five spots (0.1 ml) on a plating area comprised the counting unit and numbers are based on the average number of colonies from two counting units (10 spots).

The spot-plate method was also used to estimate surviving bacteria during disinfection tests and to study the multiplication of E. coli in the chemostat.

## DISINFECTION EXPERIMENTS

### Cleaning of glassware

All glassware which came in contact with chlorine or contained water used to dilute bacteria or disinfectants was washed carefully with water before being immersed overnight in a solution of 5%  $\text{HNO}_3$  and 95%  $\text{H}_2\text{SO}_4$  as recommended by Kavanagh (1960). This cleaning solution was preferred to the commonly used chromic acid,





the ions of which cannot readily be removed from sintered glass filters.

After the acid treatment the glassware was rinsed with tap water, placed on racks and rinsed with hot rinse water in a washing machine. After tap water, distilled water and demineralized water rinses, the glassware was allowed to dry. Glassware and pipets used in disinfectant tests were hot air sterilized for at least 2 h at 170°C.

### Disinfection

Exposure of bacteria to chlorine was effected by mixing 10 ml of the washed bacterial suspension with 90 ml of chlorine solution. For this purpose 90 ml of buffered diluent (pH 7.2), especially prepared with distilled, demineralized water, was added to a sterile 200 ml wide mouthed Erlenmeyer flask fitted with a ground glass stopper. The flask contained a 1 1/2 in. sterile magnetic stirring rod. A volume of water was withdrawn and replaced with a quantity of the stock disinfectant solution to give any desired chlorine concentration after addition of the bacteria. The chlorine concentration of the stock solution before addition of bacteria was always checked before being used by the standard thiosulfate titration and two samples prepared in the Erlenmeyer flasks in this way were checked to make sure that organic matter introduced during the final dilution did not react with the small amounts of chlorine used in tests. The chlorine solutions were found to be surprisingly stable when kept at 20 or 32°C. The flask containing the buffered chlorine solution was placed into a



thermostatically controlled waterbath at 20 or 32°C and the temperature was allowed to equilibrate. Ten ml of a washed bacterial suspension at the temperature of the test was blown into the disinfectant solution while the magnetic stirrer was operating. After desired exposure periods, 1 ml amounts were withdrawn and transferred to 9 ml of peptone-thiosulfate diluent. These were held in an ice waterbath until spot-plates could be prepared. Ten ml samples to check metabolic damage were withdrawn and were added directly to colorimeter tubes containing 2 ml of substrate-indicator-neutralizer solution. The colorimeter tubes had been held in a waterbath to adjust the temperature of the tubes and the solution to 32°C. The tubes were closed with a 00-rubber stopper and mixed by inverting once before being returned to the 32°C waterbath for incubation.

#### Tests Using Tetrazolium Salts for Staining Bacteria

Tetrazolium salts can be converted to their colored formazans by living bacteria. In the present work tetrazolium salt was used to study the vital staining behavior of E. coli, to illustrate metabolic damage inflicted on bacteria by chlorine, and to monitor the dehydrogenase activity of an E. coli culture growing in a chemostat. The tetrazolium test for each purpose will be described below.

Preparation of substrate-solution mixtures. Substrate-solution mixtures were prepared in sterile, stoppered Klett-test tubes either by adding 0.5 ml quantities to the appropriate tubes with 1 ml pipets







or an equal volume of each of four required solutions was poured into a 125 ml flask with a ground glass neck and dispensed with a 2 ml tilt dispenser. When three substrate solutions, such as yeast extract, casein digest, and glucose, as well as neutralizer and TTC solution were required, equal volumes of double-strength neutralizer and TTC solution were mixed to give the final desired concentration. This meant that the normal 1 ml of neutralizer and TTC solution could be added by adding only 0.5 ml of a neutralizer - TTC solution made by mixing double-strength solutions. The tubes containing substrate-solution mixtures were stored in the 32°C waterbath till needed.

The effect of tetrazolium salt on formazan formation by *E. coli*.

To study the effect of tetrazolium salt concentration on formazan formation by *E. coli*, washed bacteria were incubated in the presence of different concentrations of the salt. The substrate consisted of 1 ml of complete broth and 1 ml of a proper concentrated TTC solution was added to each tube. Eight (or 10) ml of the washed bacterial suspension containing  $10^7$  cells/ml as determined colorimetrically was added/tube and the final tetrazolium concentration for the whole volume was calculated. A tube of substrate without bacteria was always prepared and incubated to make certain that the substrate did not result in chemical reduction of tetrazolium salt. In the blank the buffered tetrazolium salt was replaced by buffered water and the blank was incubated with the other tubes. The blank was used to adjust the colorimeter to zero and to compensate for the turbidity of the tube. The optical density of a tube containing tetrazolium salt represented



the amount of formazan formed by the bacteria in the tube. The colorimeter was used with filters no. 42 or 52 for this series of experiments and this is specified.

Tubes in all tetrazolium tests were incubated in a waterbath at 32°C under artificial light from a 150 W Sylvania white light bulb suspended centrally about 2 feet above the waterbath, since enzymic formazan formation appears to occur mainly in the light (Jámbor, 1954).

Tetrazolium test for monitoring E. coli growing in the chemostat. Approximately 50 ml of the culture was withdrawn with a syringe from the growth vessel of the chemostat. Ten ml amounts were added to 2 ml substrate-solution mixtures and the tubes were incubated at 32°C for 20 min. The tetrazolium concentration in the reaction tubes was 200 ppm and the amount of formazan was determined colorimetrically by using the appropriate blank and compensating for the turbidity of the tubes. A no. 42 filter was used in the colorimeter.

Illustration of metabolic damage by chlorine. To colorimeter tubes containing one or two substrates (1 ml), 0.5 ml of neturalizer solution and 0.5 ml of a 4800 ppm buffered tetrazolium salt solution (to give a final salt concentration of 200 ppm) were added 10 ml of the bacteria-disinfectant suspension. The tubes were stoppered, mixed and returned to the waterbath for incubation which usually lasted for 2 h. The blank was used to set the colorimeter to zero and the amount of formazan formed was measured. A no. 42 filter was used in the colorimeter.





### Conclusion to Materials and Methods

The foregoing pages describe the major procedures used in this investigation. At times it became necessary to alter the procedure slightly to illustrate a certain specific point. Such alterations were usually minor and difficult to describe out of context with the experiment, therefore, such instances are noted in the appropriate sections.





## RESULTS AND DISCUSSION

### PRESENTATION OF RESULTS

In this section the data and relevant discussion are presented in close proximity. The data generally have been examined graphically in this section and the detailed data of experiments are presented in the Appendix. A cross reference on each of the graphs indicates the table in the Appendix containing the appropriate data and vice versa. Generally no reference is made to data in the Appendix; however, in a few isolated cases where this is necessary this is clearly indicated. Data which are not suitable for graphic presentation are presented in this section in the form of tables.

### VITAL STAINING WITH TETRAZOLIUM SALT

A few initial experiments indicated that vital staining with TTC was a rather variable phenomenon, depending on the growth history of the bacterial culture, the concentration of TTC and bacteria and utilizable substrate as well as the incubation time. It is now clear that vital staining with TTC is subject to the laws governing disinfection since the soluble, colorless tetrazolium salts are toxic; however the reduction of TTC leads to the formation of less toxic, colored, water-insoluble formazans and this may be regarded as a detoxification phenomenon. Further, it is important to remember that the conversion of TTC to formazan is dependent on enzyme reactions. This means that the vital staining phenomenon is subject to enzyme kinetics and enzyme



(bacteria), substrate concentration and incubation time are important. In making comparisons between amounts of formazan produced (optical density) in various experiments factors mentioned above should be considered. Dye reduction depends on enzymatic activity of the bacteria and the amount of formazan produced by a certain concentration of bacteria may vary with their physiological condition. It is generally not advisable to relate formazan production to bacterial numbers per se; however, if conditions are carefully controlled or if samples are drawn from a single culture, then formazan formation can be used to estimate numbers of viable bacteria or enzymatic damage with good reproducibility. The precision of the test is not so great that cultures from different days can be expected to give precisely reproducible results. To determine the reliability of the TTC test for estimating metabolic damage of bacteria by disinfectants, it appeared necessary to study the vital staining reaction of E. coli with TTC.

To avoid confusion the following convention is adopted in presenting the data: 'Optical density' or 'optical density (formazan)' is used when the OD reading refers to production of formazan, in which case a subtraction for the absorbency of the blank has been made. 'Turbidity' is used to designate the absorbency of a suspension caused by the presence of bacteria alone and is represented by an OD reading. The legends at the bottom of graphs or the headings of columns in tables are appropriately labelled.





### Estimation of Viable Bacteria by Extracting Formazan

Most workers who have used TTC for estimating dehydrogenase activity in bacteria and animal tissues have extracted the deposited formazan by organic solvents before estimating the optical density; however, Nachlas et al. (1960) observed that formazans may be kept finely dispersed for colorimetry in the reaction mixture by the addition of gelatin. Deguchi (1964) similarly noted that Tween 80 can be used to prevent precipitation of formazans. Since there was no apparent clumping of E. coli in the presence of TTC and the formazan appeared to remain finely dispersed throughout the tube, it was felt by the author that extraction of formazan from the bacteria might not be necessary in the present work, if it could be shown that changes in turbidity caused by the low concentrations of lethal agents used were not great. Some experiments in this investigation were therefore done by adjusting the background turbidity of the bacterial suspension by using a portion of the suspension after it had been sterilized in the autoclave for 15 min at 121°C.

It was of further interest to see whether bacteria could be concentrated on Millipore filters and stained on the filters for microscopic examination. These experiments were abandoned when it was noted that thin films of bacteria will not stain with TTC. Weibull (1953), also Seligman and Rutenburg (1951) had earlier noted that thin tissue sections ( $8\mu$ ) failed to stain completely. Glick and Nayyar (1956) had to submerge tissue sections on slides in the substrate



mixture for staining. More recently it has been possible to get E. coli to stain when filtered from broth on Millipore filters and washed with water. Staining of the bacterial films on filters was possible when the filter membrane was placed on a Whatman filter which had been soaked in the substrate-TTC mixture. The filters were placed on a glass plate on a slide dryer at 32°C and a second glass plate placed on top of the filters. Preliminary experiments appeared promising; however, the method was not extensively investigated.

The following procedure was used to illustrate the relationship between formazan formation and bacterial concentration shown in Fig 3. A washed bacterial suspension was diluted with buffer and the turbidity of 10 ml quantities of the dilution series was recorded colorimetrically. The 10 ml of bacterial suspension was filtered through a filter membrane (Millipore bacteriological filter type DA with an average pore diameter of  $0.65\mu$ ). The filter was rolled and placed into a sterile, empty, stoppered Klett test tube and held in an ice water bath. After all dilutions had been filtered, 2 ml of complete broth (substrate) and 1 ml of buffered TTC solution were introduced into each tube. The substrate-TTC solution had to cover the filter completely to assure efficient staining. The tubes were incubated at 37°C for 20 min. The formazan was extracted by the method of Sunila and Parmala (1955) with 3 ml of acetone for about 15 min followed with 6 ml of butanol for 5 min. Five ml of the formazan-butanol extract on the top was pipetted off to determine the optical density. The blank consisted of 10 ml dilution water treated in the same manner.





A more rapid system for extracting formazan was used for a second experiment. After incubation, 4 ml of chloroform was added to each tube. The tube was shaken gently and 5 ml of isopropynol was added. Extraction of formazan by this method occurred as quickly as the addition of the solvent. Since formazan is soluble in chloroform, it is interesting to note that none of the intracellular formazan formed by E. coli cells could be seen in the chloroform after gentle shaking; after addition of isopropynol, the formazan appeared in the chloroform-isopropynol layer at the bottom of the tube almost instantly. This observation may have application for estimating external and internal deposits of formazan. Five ml of the chloroform extract containing the formazan was used to estimate the optical density.

Fig. 3 shows that formazan production is closely related to bacterial concentration. Deviations in the curves were probably a result of difficulties experienced in extracting formazan from bacterial clumps which formed when bacteria were deposited on to filters. Other experiments, which are not presented, showed that the strain of E. coli used in this work did not clump when in suspension. Extraction of formazan from stained bacterial suspensions was usually complete when 2 to 3 ml of the stained suspension was extracted as described above. E. coli did not appear to clump when stained in suspension and since low concentrations of chlorine to be used in later experiments did not appear to result in noticeable changes in the turbidity of a bacterial suspension, it was decided that extraction of formazan was not necessary in the present investigation. However, with certain organisms, such as





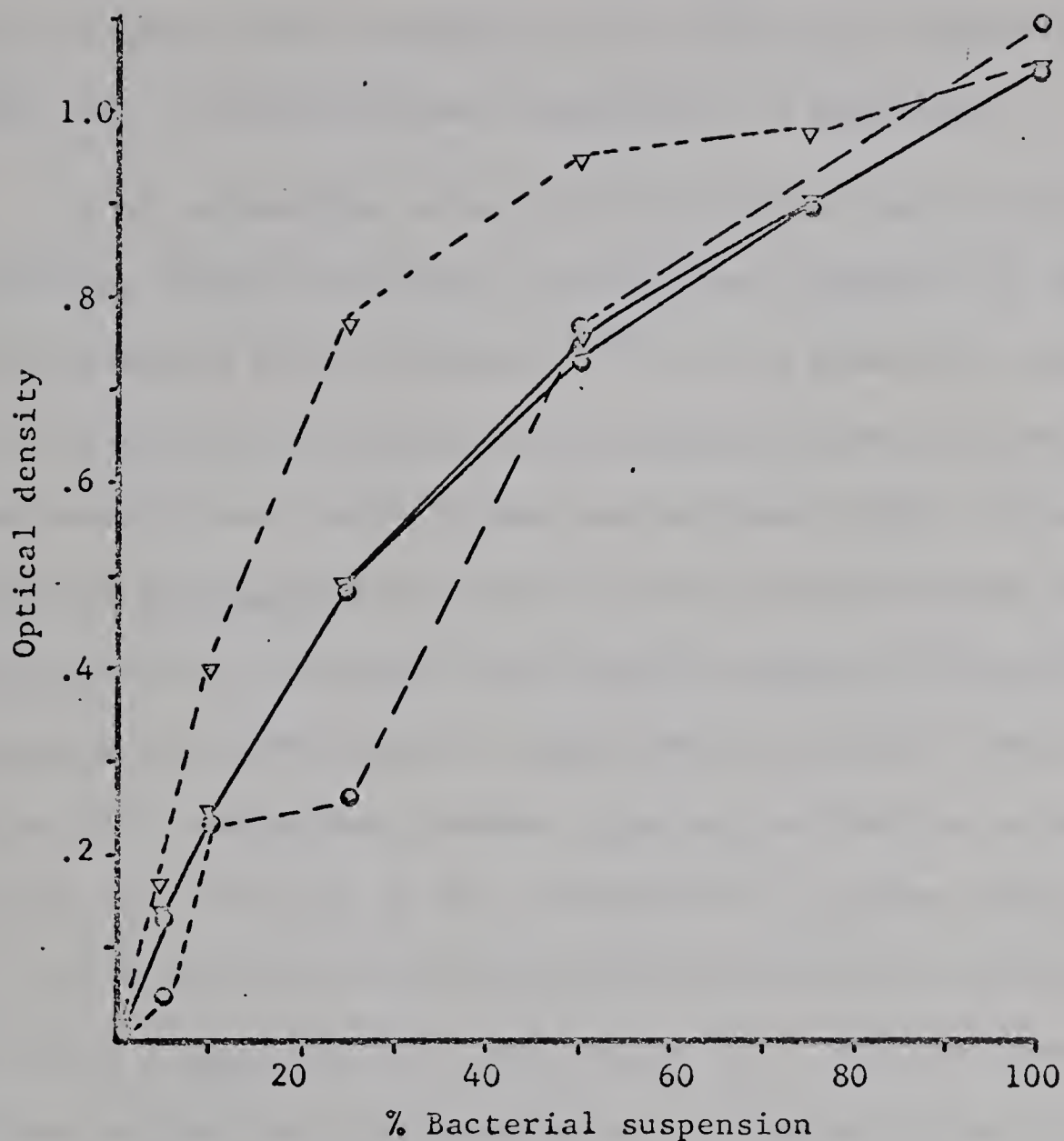


Fig. 3. The relationship between formazan production and bacterial concentration as illustrated by comparing turbidity of bacterial suspensions and optical density of solvent extract of formazan. (See Appendix, Table 1)

Turbidity of dilutions of bacterial suspensions:

Suspension (a), ●—●; (b) ▽—▽ .

Optical density of formazan extracts: Suspension (a) extracted with acetone and butanol, ●---● ; Suspension (b) extracted with chloroform and isopropynol, ▽---▽ .

Bacterial suspensions were prepared by washing 24 h (32°C) complete broth batch cultures of E. coli and optical density readings were obtained colorimetrically using a no. 42 filter.

The final TTC concentration in the experiment extracted with acetone and butanol was 800 ppm, whereas 500 ppm TTC was used when formazan was extracted with chloroform and isopropynol.



the capsulated Aerobacter aerogenes used later, dispersion with gelatin or extraction of formazan with solvents would be required, since that organism had a tendency to form clumps and adhere to the glass when a tetrazolium salt was added to a suspension.

It is appropriate to mention here that it had been observed that turbidity changes resulting from chlorine treatments of washed E. coli suspensions of approximately  $10^9$ /ml were generally slight when concentrations of chlorine not exceeding 100 ppm were used. This has recently been noted by Rode and Williams (1966) who worked with Bacillus megaterium and showed that high concentrations of sodium hypochlorite (>250 ppm) can be used to dissolve bacterial cells. Spore suspensions showed greater reductions in turbidity than did vegetative cell suspensions; however, the rate of decline in turbidity appeared to be a function of the concentration of sodium hypochlorite. Since it had been observed repeatedly that no change in turbidity resulted when E. coli cultures were treated with chlorine concentrations of <10 ppm, extraction of formazan prior to colorimetry was not used in the present investigation. Appropriate blanks were used to adjust the colorimeter to zero to compensate for the turbidity due to bacterial suspensions and substrate, and optical density readings represent formazan produced.

#### The Effect of Culture History and TTC Concentration on Vital Staining of E. coli

It had been noted that E. coli cultures grown in complete broth usually stained well with TTC; however, cultures grown in minimal





broth stained poorly. It was therefore decided to compare the staining characteristics of washed bacterial suspensions with comparable turbidity but different culture histories. For this purpose one sample was drawn from the chemostat which had been operating on minimal broth (MB) for 160 h and the other suspension was prepared from a complete broth (CB) batch culture grown at 32°C for 24 h.

After washing, the turbidity of both bacterial suspensions was adjusted to an OD of 0.460, using a no. 52 filter. The viable counts of the suspensions were found to be  $2.3 \times 10^8$ /ml for the MB culture and  $9.6 \times 10^7$ /ml for the CB culture before 8 ml of the bacterial suspension was added to 2 ml of substrate-TTC mixture ( $1.8 \times 10^8$ /ml and  $7.7 \times 10^7$ /ml respectively after dilution, see Table 2, Appendix).

Fig. 4 clearly shows that formazan production is affected by both culture history of E. coli and the TTC concentration. The amount of formazan formed by the bacteria grown in complete broth tends to be slightly higher than the formazan produced by bacteria harvested from minimal broth, even though the bacterial concentration in numbers was greater for the MB culture.

Formazan formation increased with increasing TTC concentration to approximately 600 ppm when the inhibitive effect of TTC is evident with both bacterial suspensions. It is interesting to note that the inhibitive effect of TTC occurs at the same concentration for both suspensions and this probably indicates that the inhibition is not solely related to bacterial numbers, but that the concentration of



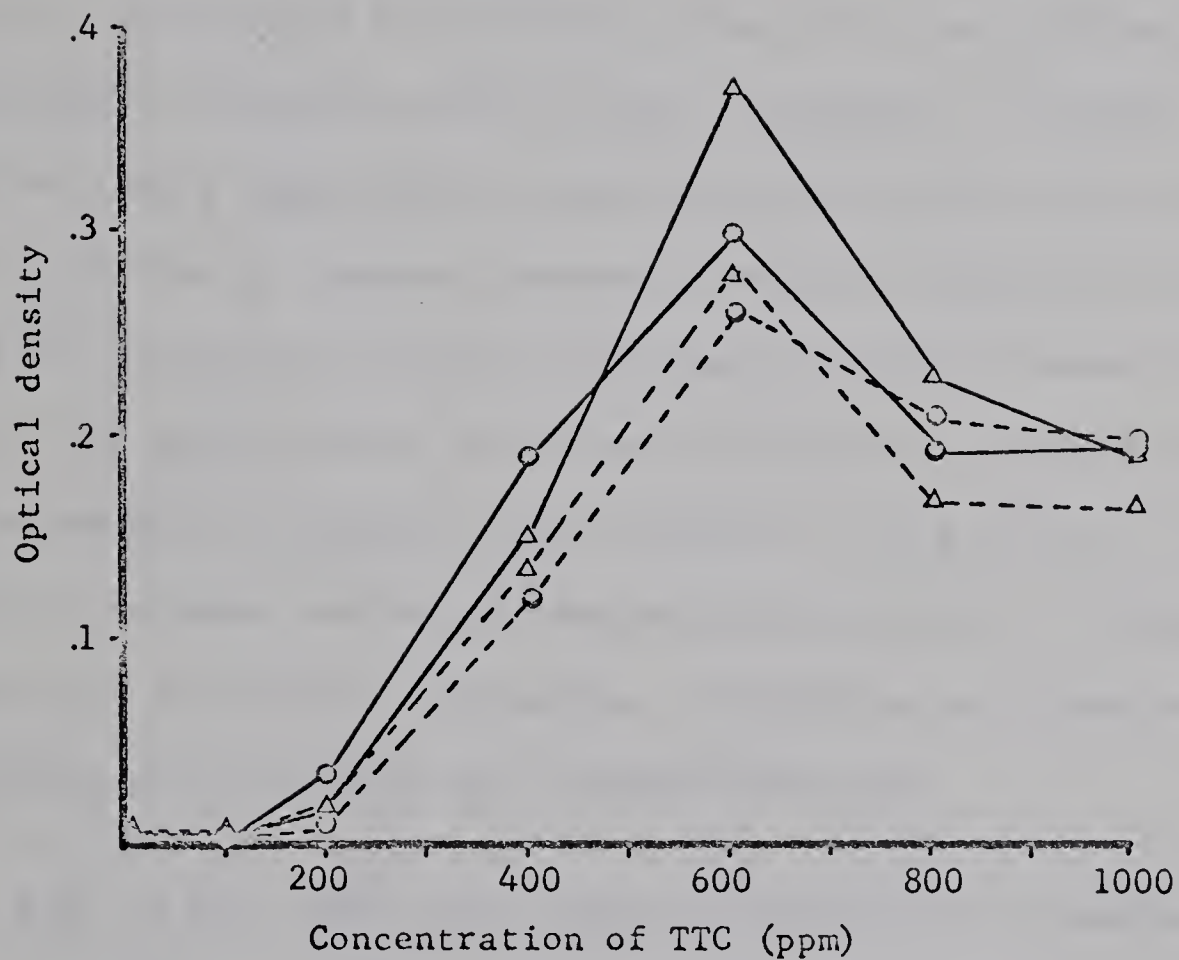


Fig. 4. The effect of TTC concentration on vital staining of *E. coli* with different culture histories. (See Appendix, Table 2). (Minimal broth (MB) culture incubated at 32°C for: 60 min ●---●, 120 min ●—●; complete broth (CB) culture incubated at 32°C for: 60 min ▲---▲, 120 min ▲—▲.) The colorimeter was equipped with a no. 52 filter and washed bacterial suspensions of comparable turbidity were prepared from a 160 h minimal broth chemostat culture and a 24 h complete broth batch culture.



bacterial proteins is involved. It could be noted microscopically that cells grown in minimal broth were definitely smaller than those grown in complete broth.

Further experiments illustrating the effect of culture history, bacterial and TTC concentrations on formazan production are given in Figs. 5, 6, 7a and b. Fig. 5 shows that the E. coli culture grown in the chemostat with minimal broth produced the greatest amount of formazan at 400 ppm TTC when the incubation time is 30 and 60 min and when the concentration of bacteria is sufficient so as not to be effected by the disinfectant properties of TTC (Table 3, Appendix). However Fig. 6 shows that E. coli grown in complete broth batch culture did not show inhibition of formazan production when the bacterial concentration was  $1.8 \times 10^8$  bacteria/ml and TTC concentration varied between 100 and 1000 ppm. This graph further shows that inhibition of formazan production was pronounced when the bacteria were diluted to  $1.8 \times 10^7$ /ml. In fact production of formazan was delayed and measurable amounts of formazan were noted only after 90 min incubation. Inhibition was strong at concentrations of tetrazolium salt exceeding 600 ppm.

Fig. 7a once again shows that no inhibition of formazan production can be noted with E. coli grown in batch culture in complete broth when the cells were present at a concentration of  $2.6 \times 10^8$ /ml; however, the amount of formazan produced increases in relation to the TTC concentration (Fig. 7a and 7b). It can also be seen (Fig. 7b) that formazan is largely produced during the first 60 min of incubation.





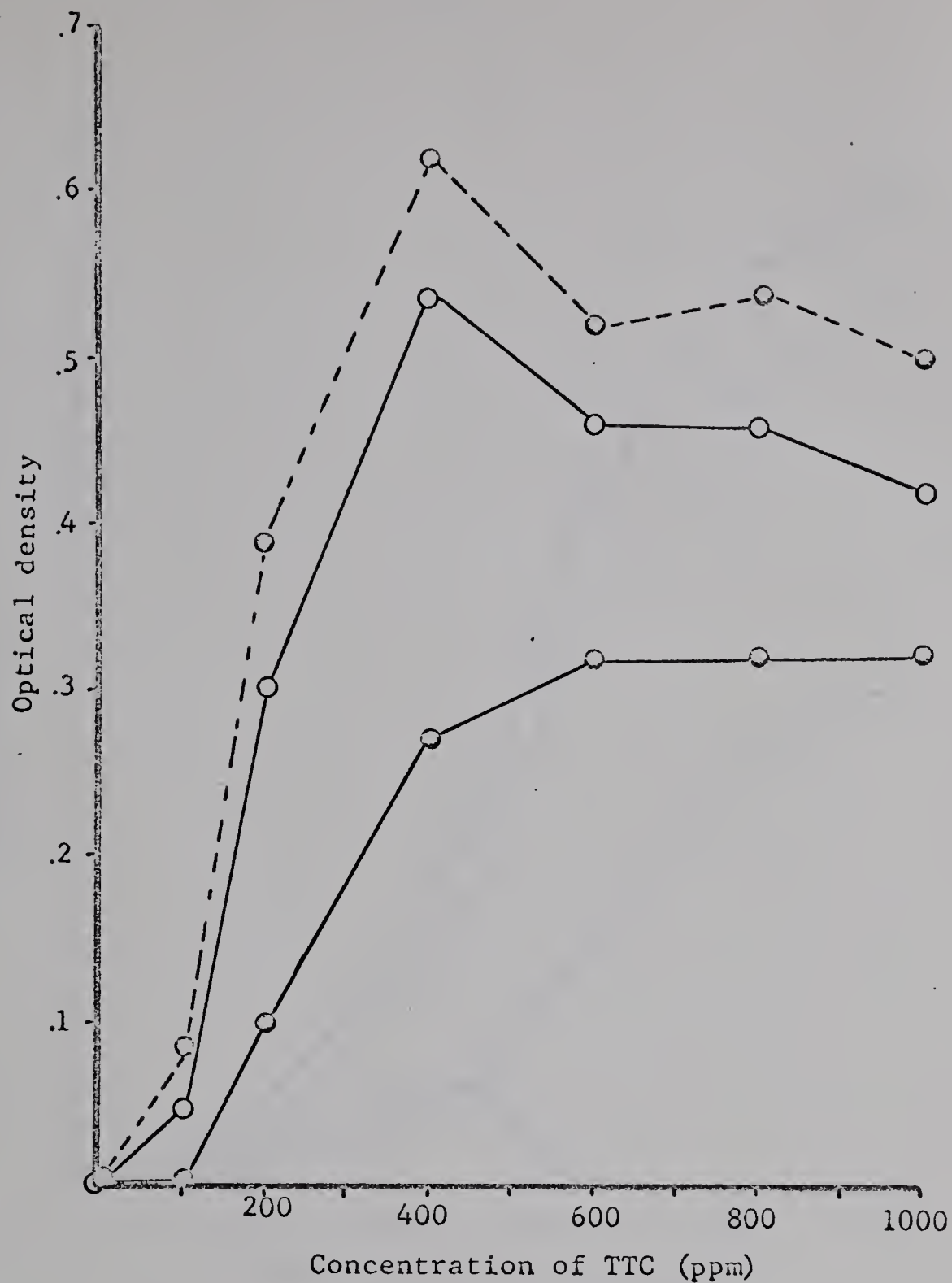


Fig. 5. The effect of TTC concentration on vital staining of *E. coli* maintained in the chemostat. (See Appendix, Table 3). ( $7.4 \times 10^8$  bacteria/ml incubated at  $32^\circ\text{C}$  for: 10 min  $\bullet\text{---}\bullet$  ; 30 min  $\circ\text{---}\circ$  ; 60 min  $\bullet\text{---}\bullet$  .) The colorimeter was equipped with a no. 52 filter and the bacterial suspension was prepared from a 190 h minimal broth chemostat culture.



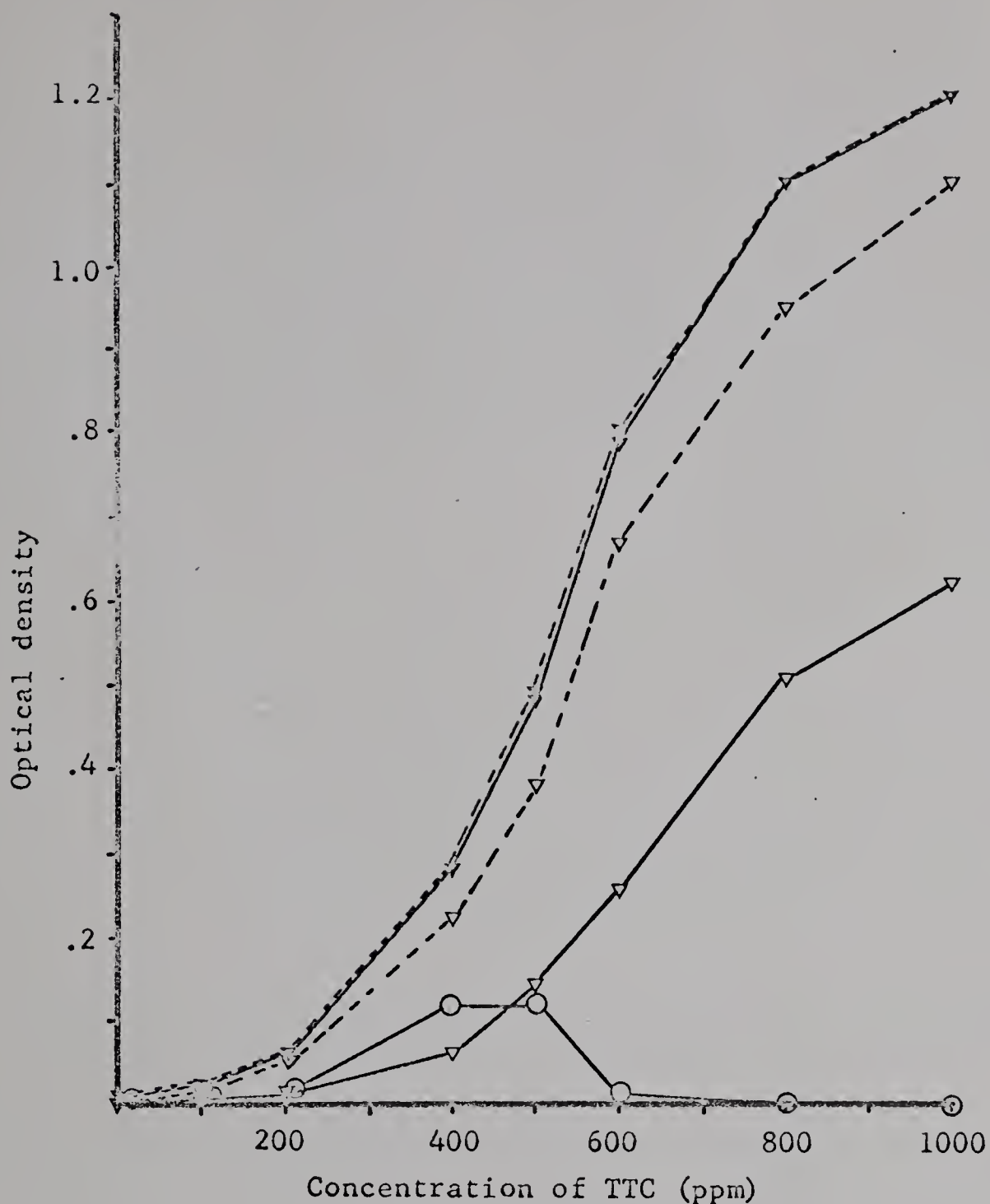


Fig. 6. The effect of TTC concentration on vital staining of *E. coli* grown in batch culture in complete broth. (See Appendix, Table 4).  
 ( $1.8 \times 10^8$  bacteria/ml incubated at  $32^\circ\text{C}$  for 10 min ▽—▽ ; 30 min ▽---▽ ; 60 min ▼—▼ ; 90 min ▼----▼ .)  
 ( $1.8 \times 10^7$  bacteria/ml incubated at  $32^\circ\text{C}$  for 90 min ○—○ .)  
 The colorimeter was equipped with a no. 52 filter and the bacterial suspension was prepared from a 24 h complete broth batch culture.

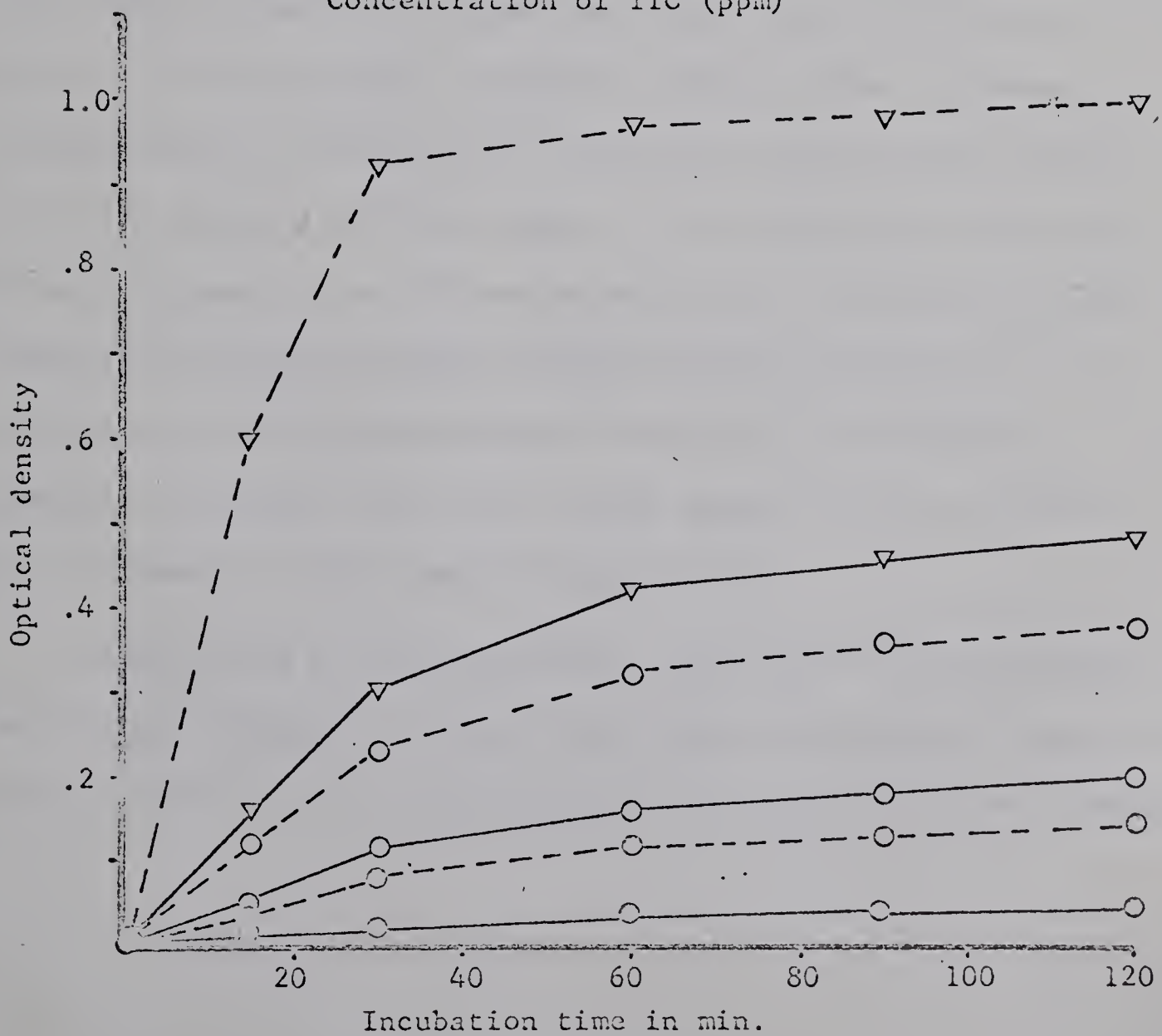
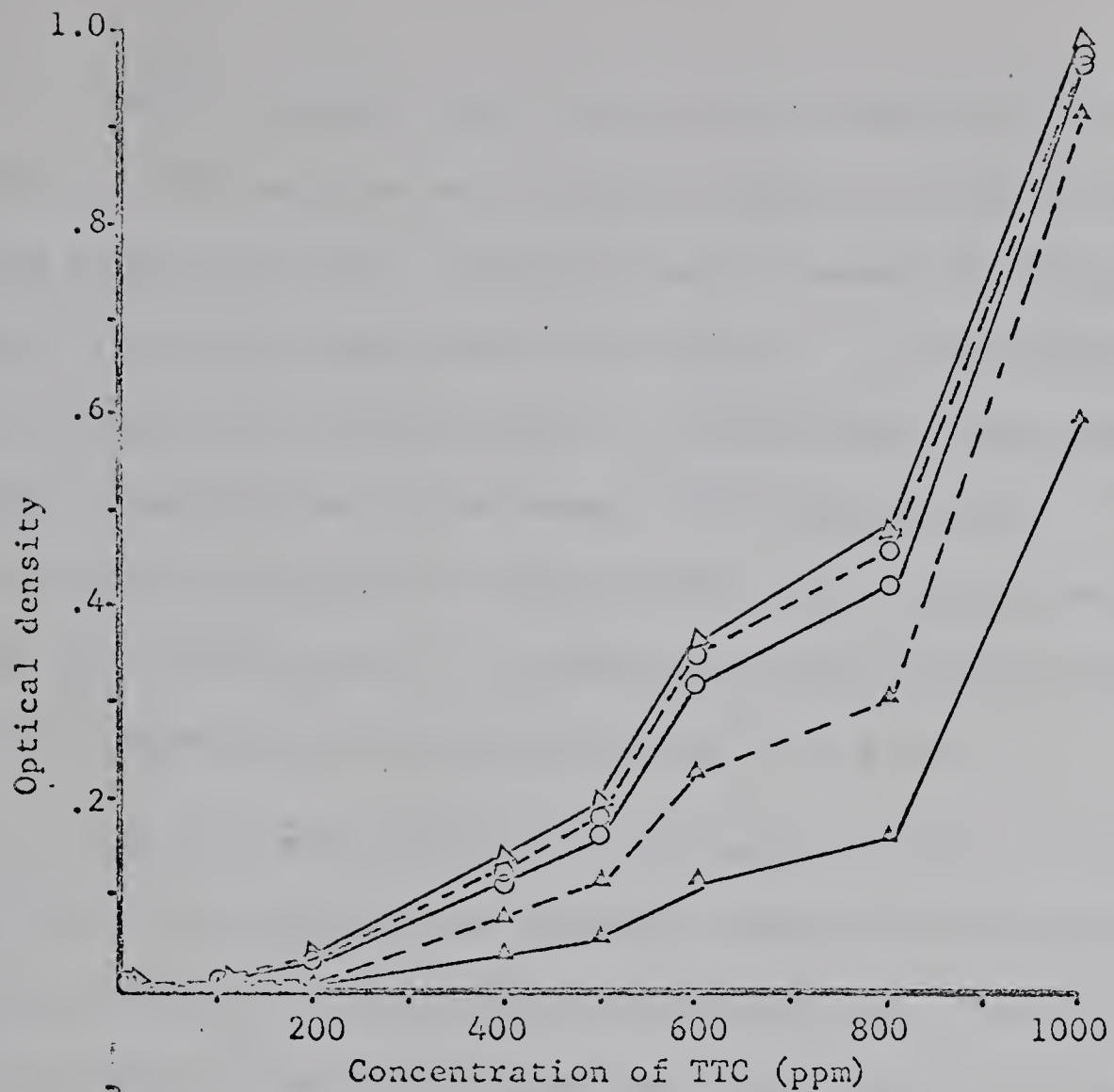






Fig. 7a. The effect of TTC concentration on vital staining of E. coli grown in batch culture in complete broth. (See Appendix, Table 5).  
( $2.6 \times 10^8$  bacteria/ml incubated at  $32^\circ\text{C}$  for 15 min  $\blacktriangle$ — $\blacktriangle$  ; 30 min  $\blacktriangle$ ----- $\blacktriangle$  ; 60 min  $\circ$ — $\circ$  ; 90 min  $\circ$ ----- $\circ$  ; 120 min  $\triangle$ — $\triangle$  .)  
The colorimeter was equipped with a no. 52 filter and the bacterial suspension was prepared from a 15 h complete broth batch culture.

Fig. 7b. The effect of incubation time on vital staining of E. coli grown in batch culture in complete broth.  
( $2.6 \times 10^8$  bacteria/ml incubated at  $32^\circ\text{C}$  with TTC at: 200 ppm  $\bullet$ — $\bullet$  ; 400 ppm  $\bullet$ ----- $\bullet$  ; 500 ppm  $\circ$ — $\circ$  ; 600 ppm  $\circ$ ----- $\circ$  ; 800 ppm  $\nabla$ — $\nabla$  ; 1000 ppm  $\nabla$ ----- $\nabla$  .)  
(Data from Table 5 Appendix.)





Another question which had to be considered, dealt with the influence of dead bacteria on formazan production by live bacteria. For this purpose bacterial suspensions were prepared by mixing suspensions of live and dead (autoclaved) bacteria. This procedure was used to simulate the various stages in a disinfectant experiment, and bacterial concentrations are expressed as "% live bacteria". Experiments using bacteria grown in complete broth batch culture are illustrated in Figs. 8a, b and 9a and b. An experiment using bacteria from a minimal broth fed chemostat is illustrated in Figs. 10a, b and c.

Fig. 8a clearly shows that low concentrations of bacteria (20 and 40% live bacteria) were adversely affected at TTC concentrations exceeding 500 ppm and TTC reduction was slowed down. On the other hand, the 100% live bacterial suspension showed increased formazan formation as TTC concentration increased. Fig. 8b shows the shape of curve demonstrated by TTC reduction that can be expected when bacteria die due to the action of a lethal agent. It is evident that the shape of the curve depends on the TTC concentration i.e. 300 ppm and 500 ppm give decelerating curves probably because TTC is a limiting factor of the conversion at high concentrations of bacteria, and 700 ppm is approximately a straight line since a large amount of TTC can be converted to formazan by high levels of live bacteria.

Figs. 9a and b are essentially a repetition of the experiment graphed in Figs. 8a and b. It can be seen that inhibition of formazan production is evident with low concentrations of bacteria (10%) but formazan





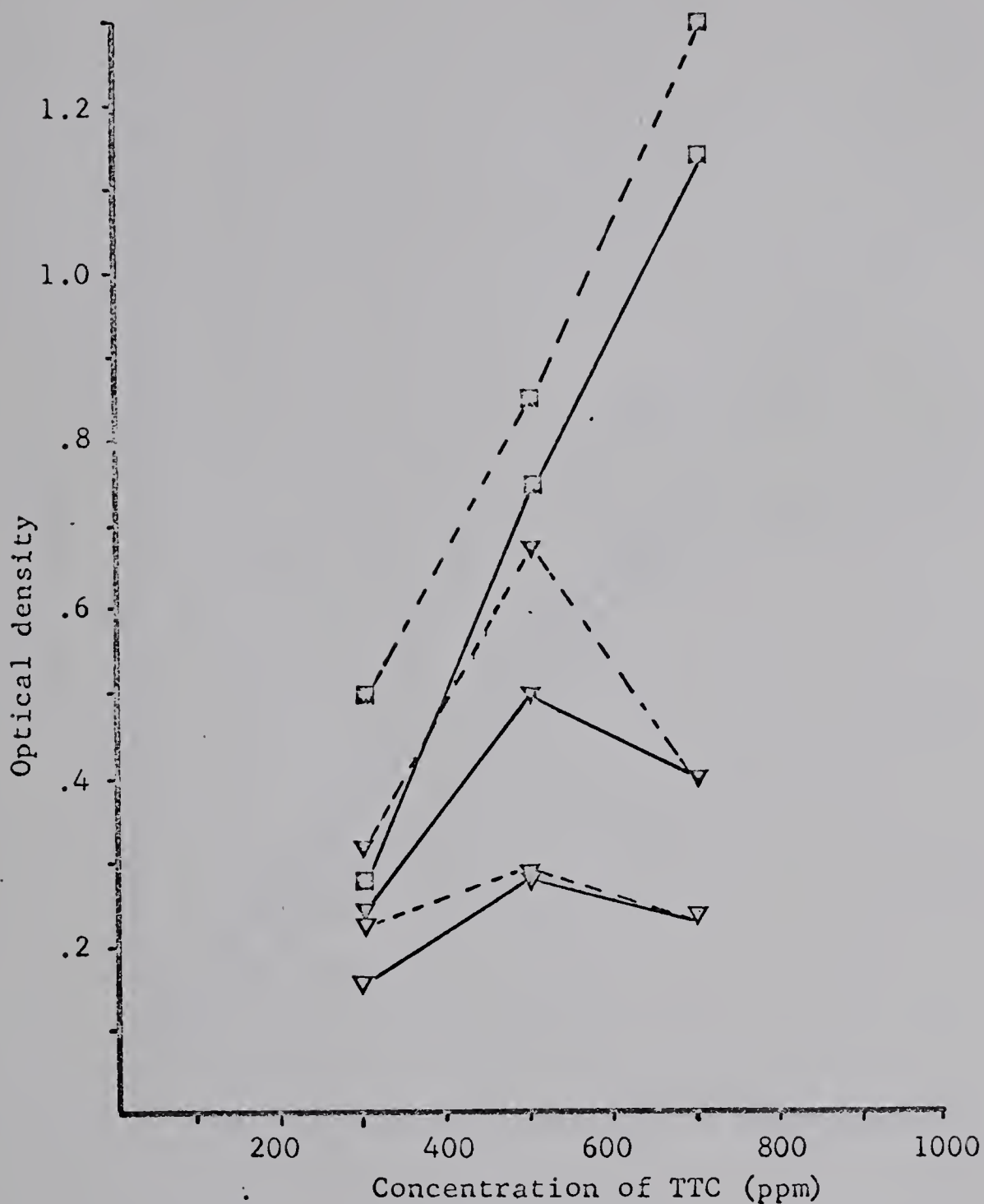


Fig. 8a. The effect of staining *E. coli* grown in complete broth in batch culture for 20 h at 32°C in the presence of dead bacteria (See Appendix, Table 6).  
(Incubation time at 32°C for 90 min — ; 180 min - - - - ;  
% live bacterial suspension: 100 ■ ; 40 ▼ ; 20 ▴.)  
The colorimeter was equipped with a no. 42 filter which was used for all subsequent experiments.



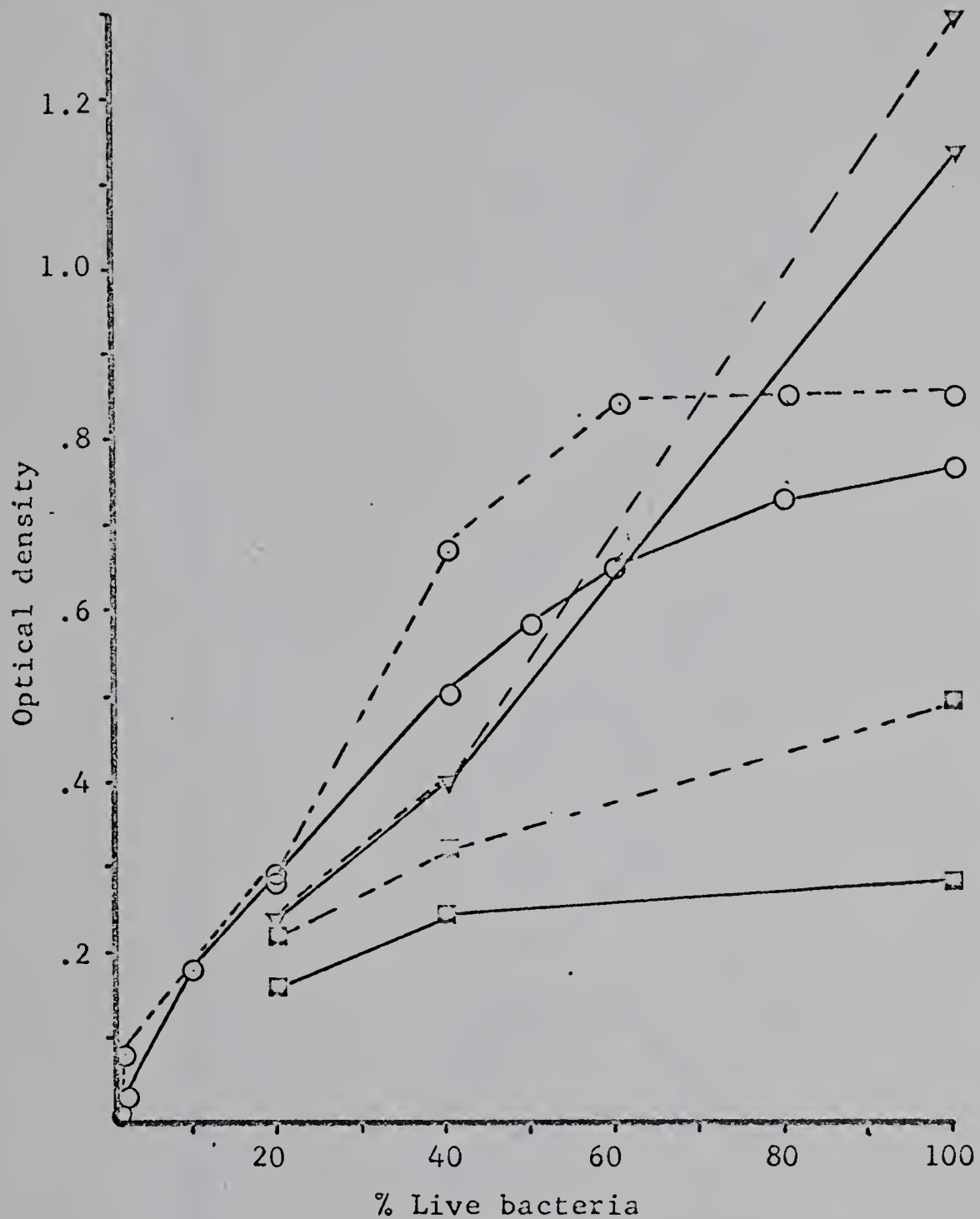


Fig. 8b. The effect of bacterial concentration on vital staining with different TTC concentrations. (See Appendix, Table 6). (Incubation time at 32°C for 90 min ——— ; 180 min ----- ; concentration of TTC: 300 ppm ■ ; 500 ppm ○ ; 700 ppm ▼.)





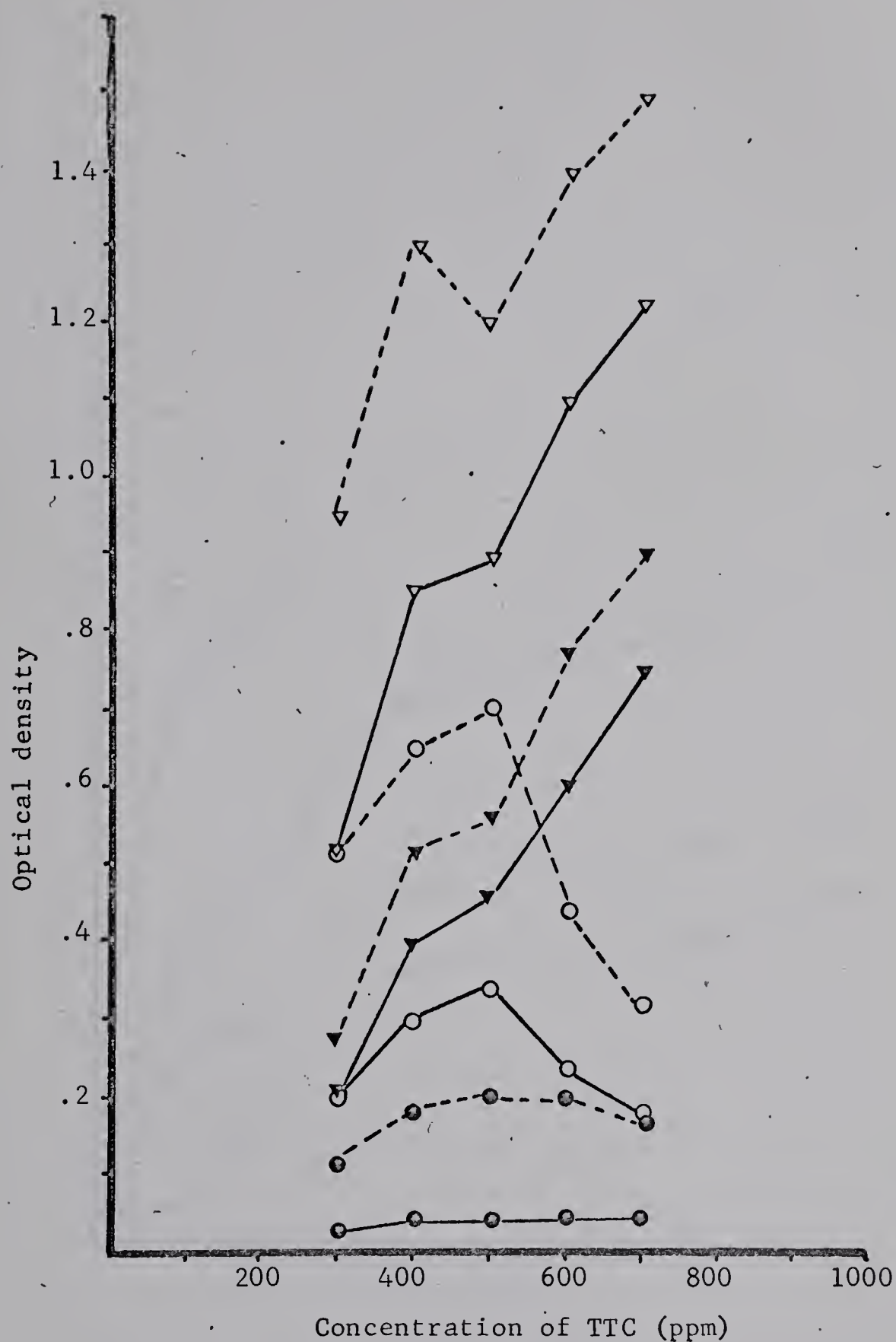


Fig. 9a. The effect of bacterial, TTC concentration and time of incubation on vital staining of *E. coli* grown for 24 h at 32°C in complete broth batch culture in the presence of dead bacteria. (See Appendix, Table 7).  
 (Concentration of live bacteria: 10%, ●, ○; 100%, ▼, ▽; incubation time at 32°C: 1 h, ●—●, ▼—▼; 2 h, ●- - ●, ▼- - ▼; 4 h, ○—○, ▽—▽; 17 h, ○- - ○, ▽- - ▽.)



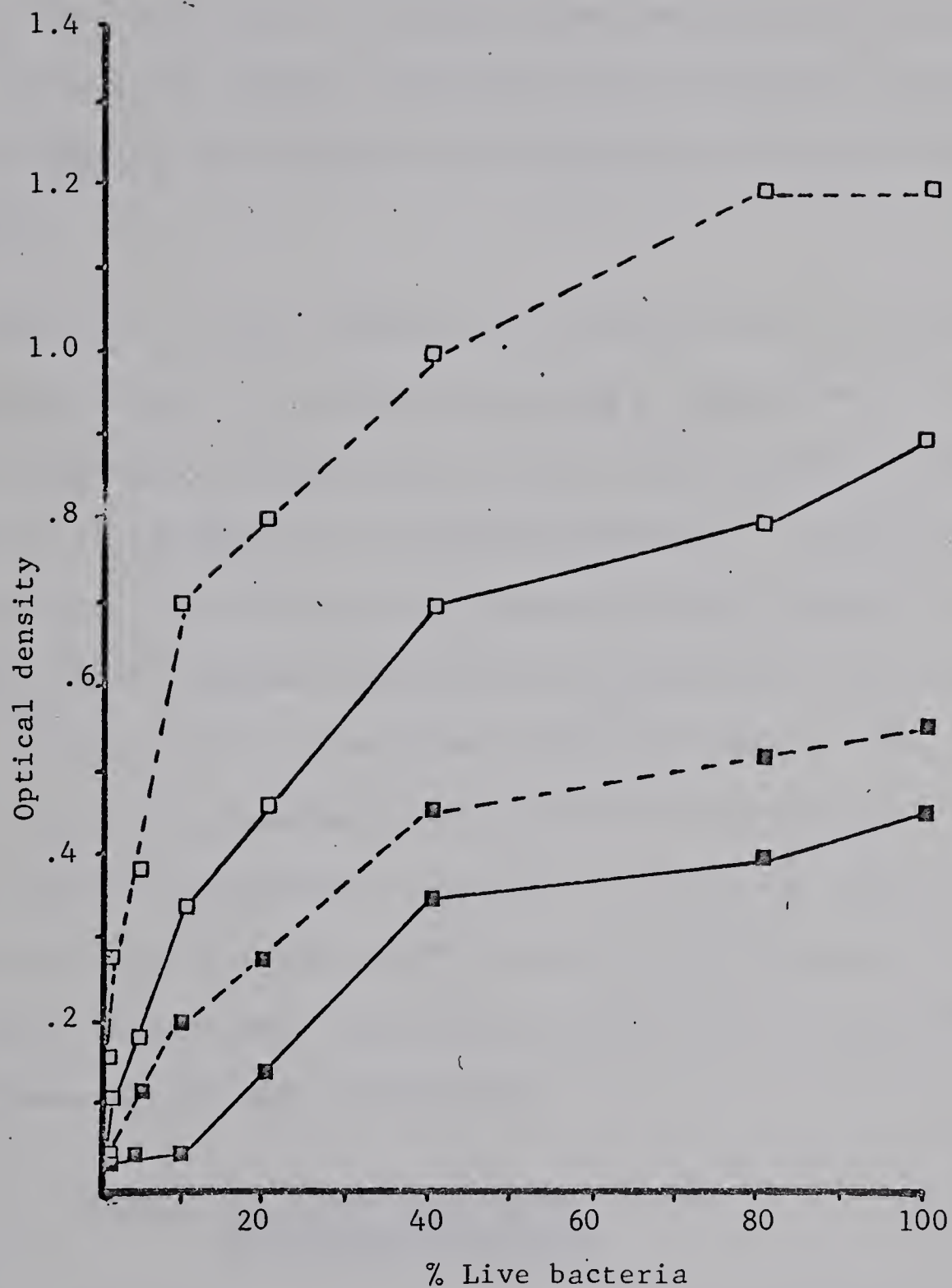


Fig. 9b. Amount of formazan produced by various concentrations of live bacteria with 500 ppm TTC. *E. coli* culture grown in complete broth batch culture for 24 h. (See Appendix, Table 7). (Incubation time at 32°C: 1 h,  $\blacksquare$ — $\blacksquare$  ; 2 h,  $\blacksquare$ ---- $\blacksquare$  ; 4 h,  $\square$ — $\square$  ; 17 h,  $\square$ ---- $\square$  .)



production increases as TTC concentration increases when the bacterial suspension consists of 100% live bacteria. It is worth noting that the shape of the curves does not change during prolonged incubation (17 h). The curve for formazan production using 500 ppm TTC can be seen to be asymptotic and remains that way when the incubation time is prolonged.

Figs. 10a, b and c explain why difficulty should be expected when attempts are made to use TTC for staining E. coli grown in minimal broth in a chemostat. Although the bacterial concentration was high (as judged by the turbidity of the washed suspension), it can be seen that maximum amounts of formazan were produced between 100 and 200 ppm TTC. Inhibition of formazan production was pronounced at TTC concentrations in excess of 200 ppm as shown in Figs. 10b and c. Fig. 10a shows that most of the formazan is produced during the first h of incubation and that the amount of formazan produced by a 100% live bacterial suspension decreases as TTC concentration increases beyond 200 ppm. This suggests that the inhibition effect of TTC represents permanent damage to the cells of E. coli.

Summary of Vital Staining of E. coli  
with Tetrazolium Salt

Tetrazolium salts are bactericidal in a manner similar to other quaternary ammonium compounds and it is therefore not surprising that vital staining with TTC is subject to the laws governing disinfection in many respects. Thus Weinberg (1953) reported that Gram-







Fig. 10. The effect of bacterial and TTC concentration and incubation time on vital staining of *E. coli* maintained in the chemostat for 281 h. The culture was maintained in the chemostat with minimal broth at a dilution rate of 0.1/h at 32°C. (See Appendix, Table 8).

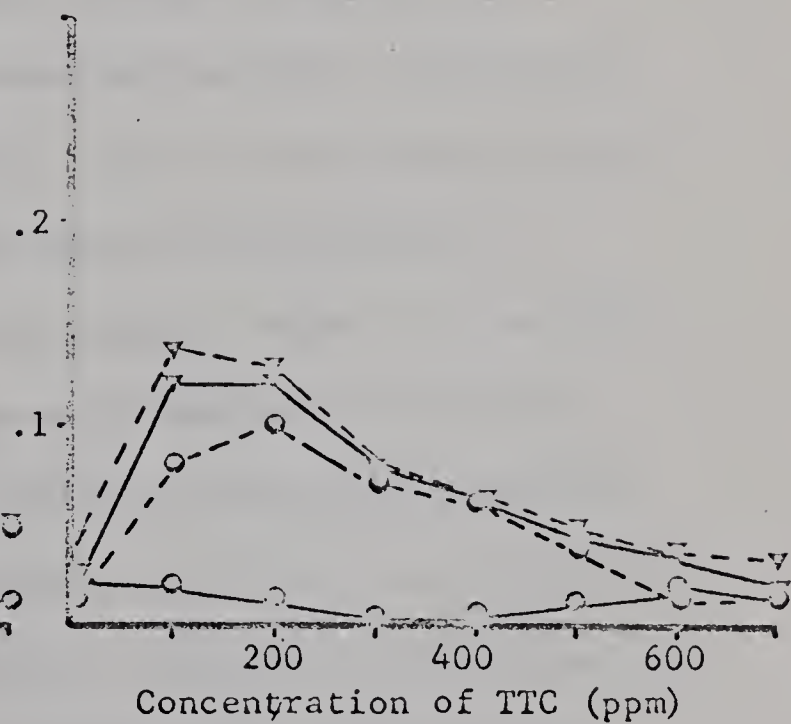
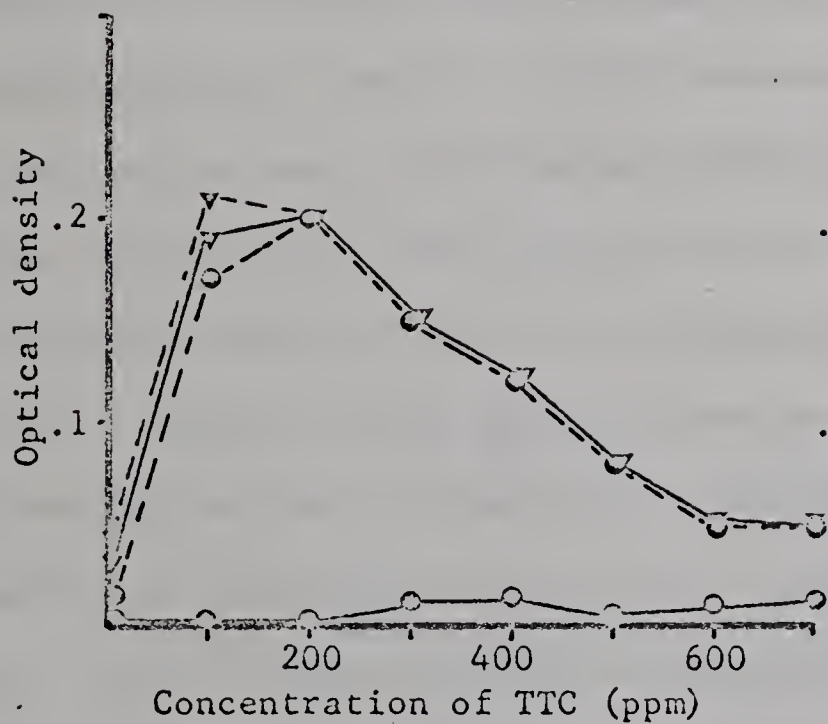
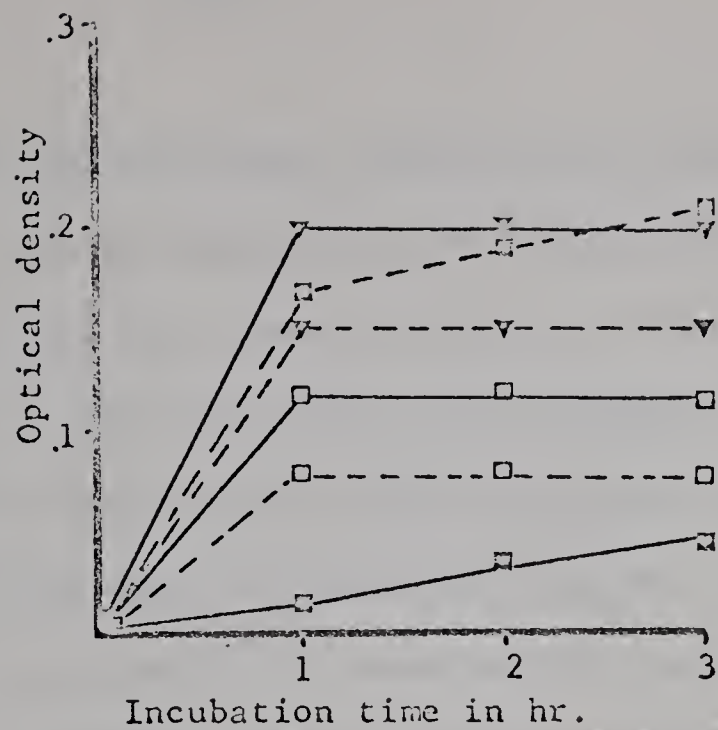
a. Effect of incubation time on vital staining of the 100% live bacteria suspension.

(TTC concentration 0 ppm ■——■ ; 100 ppm ■-----■ ; 200 ppm ▼——▼ ; 300 ppm ▼-----▼ ; 400 ppm □——□ ; 500 ppm □-----□ .)

b. Effect of TTC concentration on vital staining of the 100% live bacteria suspension.

c. Effect of TTC concentration on vital staining of the 50% live bacteria suspension.

(Incubation time at 32°C for 0 h ●——● ; 1 h ●-----● ; 2 h ▼——▼ ; 3 h ▼-----▼ .)







positive bacteria were inhibited more readily by TTC than were Gram-negative organisms. Korn and Kushnarev (1965) noted that bacteria appear to be decomposed by high concentrations of TTC and that drastic morphological changes in bacteria occur in the presence of TTC. The above mentioned results were obtained when tetrazolium salts were incorporated in bacterial media and incubation was more than 6 h. In the present work it was desired to know how TTC concentrations would affect formazan formation (that is vital staining) when the incubation was approximately 2 h.

It can be seen (Fig. 3) that formazan production is essentially a function of bacterial concentration, i.e. OD curves for the turbidity of bacterial dilutions and their formazan extracts are nearly parallel; however, the TTC concentration can have a pronounced effect on the amount of formazan produced. Fig. 4 shows that bacterial mass, as measured by OD (turbidity) is probably more important in formazan production than bacterial numbers per se. Figs. 4, 5 and 10a, b and c suggest that E. coli cultures grown in minimal broth (in the chemostat) are more susceptible to inhibition by TTC than are bacteria harvested from complete broth batch culture. Thus Figs. 6, 7a, 8a and 9a clearly show that high concentrations of bacteria ( $>10^8$  bacteria/ml) are not inhibited as TTC concentrations increase from 100 to 1,000 ppm; however, when bacterial concentrations are reduced by dilution, inhibition becomes evident even when bacteria are grown in complete broth batch culture (Figs. 4, 6, 8a, 9a). It is evident that TTC appears to poison the very enzymes (dehydrogenases) which reduce it to formazan.



An appreciation of this phenomenon is of importance in designing a TTC test for monitoring dehydrogenase damage of bacteria due to the action of lethal agents.

Whereas dead bacteria do not appear to reduce TTC (Figs. 8b and 9b) actively it was conceivable that the presence of large numbers of dead bacteria may result in interference of reduction by adsorbing TTC or by serving as additional substrate for live bacteria. Figs. 8a, b, and 9a, b and 10a, b and c show the influence of dead bacteria on vital staining of E. coli. Dead bacteria were produced by autoclaving a bacterial suspension and mixtures prepared from suspensions containing live and dead bacteria varied surprisingly little in turbidity as can be seen from Table 7 in the Appendix. It can be seen that the turbidity of bacterial suspensions increases from OD.240 to OD.260 when the % live bacteria decreases from 100% to 1%. A comparison of Figs. 6 and 7a with Figs. 8a and 9a suggests that more formazan is produced by complete broth batch cultures when dead bacteria are present. It was felt that this may be due leakage of cell material from dead cells. The cell material could serve as substrate for live bacteria, resulting in more formazan production. Substrate concentration was therefore increased in later experiments.

Many of the effects measured colorimetrically were clearly visible, i.e. an optical density reading of .030 (no. 42 filter) could be read as "pink" by visual inspection (Table 8 in the Appendix).





From these experiments it was concluded that TTC could be used to monitor dehydrogenase damage (cell death) due to various lethal agents; however, the concentration of TTC would have to be low to minimize the inhibition of formazan production and the contribution of TTC in destroying bacteria. Figs. 10a, b and c showed that E. coli suspensions prepared from chemostat grown minimal broth cultures were inhibited by TTC concentrations in excess of 200 ppm. Since studies were to be conducted with chemostat grown E. coli it was decided to use 200 ppm TTC as a concentration in subsequent work. Extraction of formazan was not thought necessary since lethal conditions to be used were not severe, however appropriate blanks were used to compensate for the turbidity of reagent tubes.

#### Maintenance of E. coli in the Chemostat

Early experiments indicated that sterility of the chemostat could be maintained for prolonged periods before inoculation of the culture vessel with E. coli. Sterility of the assembly was checked by withdrawing 10 ml of the medium with a syringe through the sampling port from the filled culture vessel while air and medium pumps were operating. The sample was filtered through a Millipore filter (0.45  $\mu$  average pore diameter) and the filter was incubated for 24 h on complete agar to confirm sterility. Samples of medium for sterility testing were also obtained from the product reservoir. After it had been proved that the chemostat could be assembled and operated without becoming contaminated, continuous culture experiments with E. coli were run.





Initial experiments were disappointing because degenerative changes were frequently noted in the E. coli culture after the steady state had been attained in the culture vessel. Poor mixing of the glucose and the buffered nutrient solution in the two stage medium reservoir was thought to be partially responsible for these changes; however, Webb (1964) noted that unexplainable degenerative changes have been reported as limiting the length of time for continuous maintenance of microbial cultures. It became apparent that great care would be required in operating the chemostat and that viable counts and turbidity readings alone were not sufficient information about the operation of the chemostat. Estimation of relative dehydrogenase activity, using TTC, on samples withdrawn from the culture vessel, soon revealed that variation in the physiological state of the culture was greater than was reflected by viable counts. Results of four separate runs are shown in Figs. 11, 12, 13 and 14. It should be noted that frequent monitoring (once every 4 h during the day and once or twice in the evening) of the runs recorded in Figs. 13 and 14 resulted in less oscillation in the steady state operation.

It can be seen from Figs. 11 and 12 that the E. coli cell concentration (viable counts) alone would have suggested steady state operation of the chemostat in both experiments. A slight increase in bacterial cell concentration occurred when the chemostat was fed with complete broth (Fig. 12) and changes in cell concentration are reflected by changes in culture turbidity. Figs. 11 and 12 further show that dehydrogenase activity measurements on culture samples using glucose plus yeast extract or glucose alone as substrates give essentially parallel graphs as long as minimal broth is fed into the chemostat and it is difficult to interpret the meaning



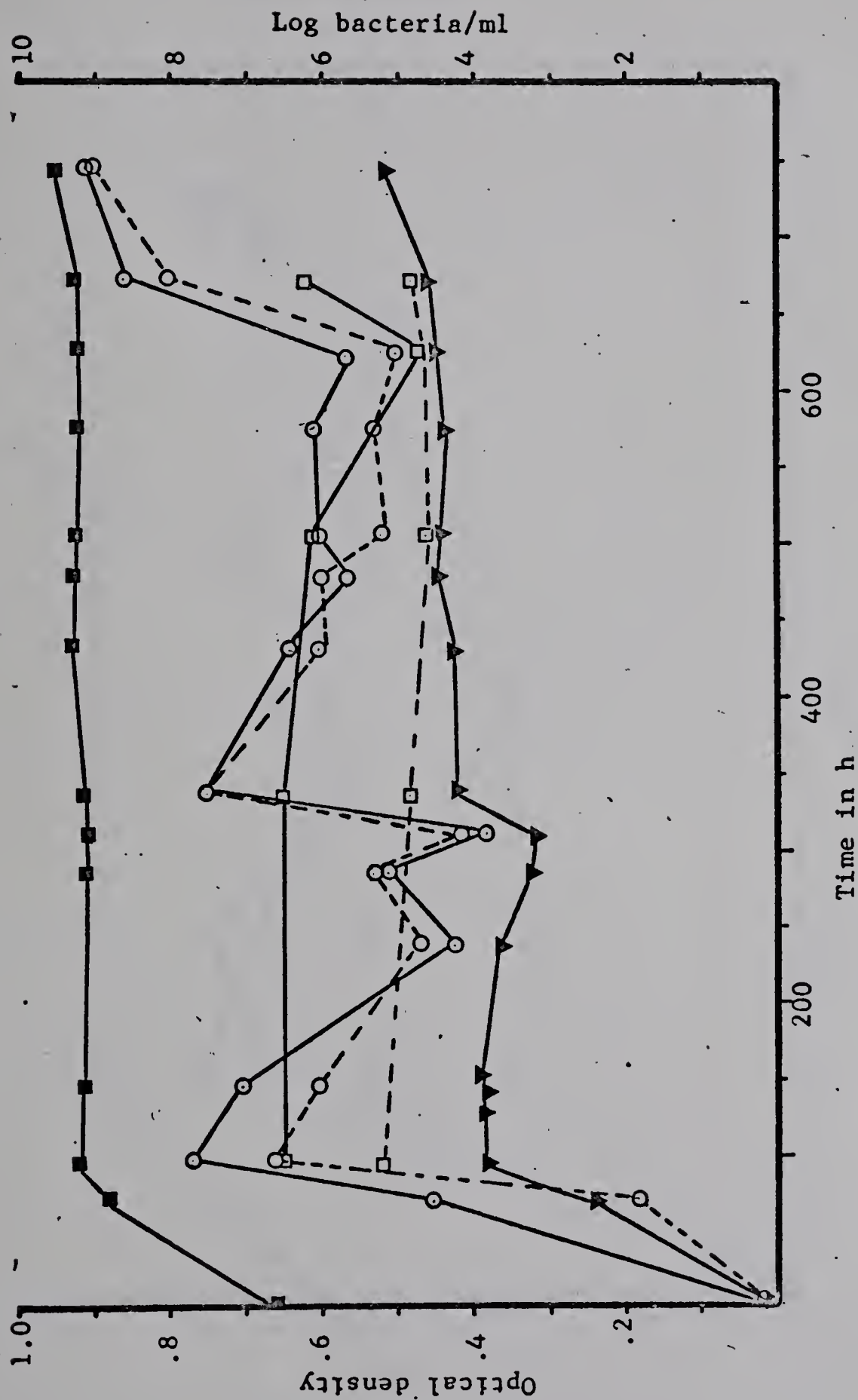


Fig. 11. Growth of *E. coli* in continuous culture with minimal medium. (See Appendix, Table 9). (The dilution rate was 0.1/h and the temperature was maintained at 32°C, except when water circulating pump failed. The air supply was quite variable. Log bacteria/ml  $\blacksquare$ — $\blacksquare$ ; turbidity of culture  $\blacktriangledown$ — $\blacktriangledown$ ; dehydrogenase activity at 32°C for 20 min of a 10 ml sample from the chemostat with glucose + yeast extract  $\bigcirc$ — $\bigcirc$ ; glucose  $\bigcirc$ — $\bigcirc$ ; dehydrogenase activity of the 1:10 dilution of the washed bacterial suspension with the turbidity adjusted to OD 1.040 and incubated at 32°C for 2 h with: glucose + yeast extract  $\square$ — $\square$ ; glucose  $\square$ — $\square$ .)





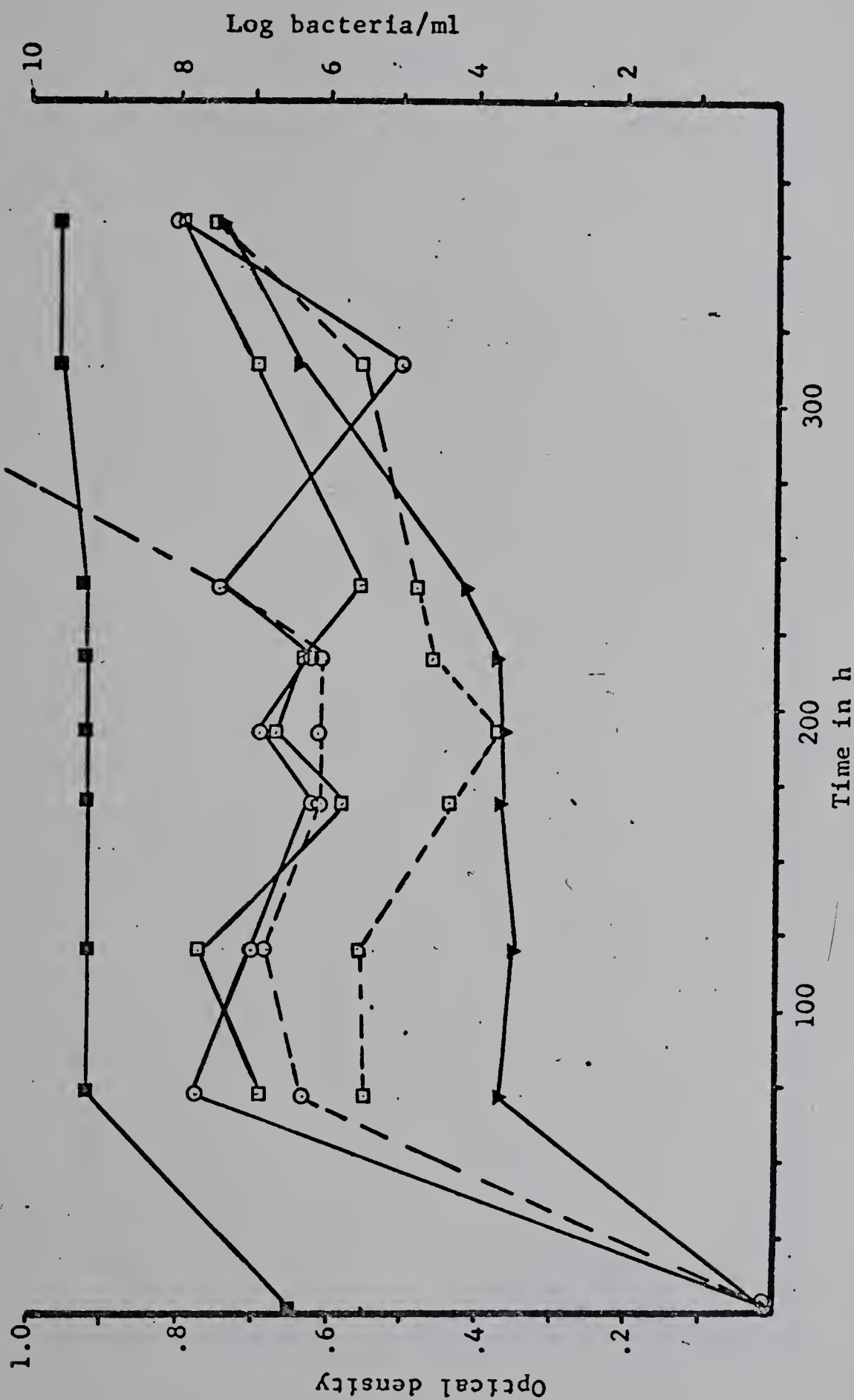


Fig. 12. Growth of *E. coli* in continuous culture in minimal medium, but complete medium introduced at 288 h. (See Appendix, Table 10).

(The dilution rate was 0.1/h and the temperature was maintained at 32°C. After 288 h of minimal broth, complete broth was fed into the culture. Log bacteria/ml

■ —■ ; turbidity of the culture —■ —■ ; dehydrogenase activity at 32°C for 20 min of a 10 ml sample from the chemostat with: glucose + yeast extract ○ —○ ; glucose ○ —○ —○ ; dehydrogenase activity of the 1:10 dilution of the washed bacterial suspension with the turbidity adjusted to OD 1.040 and incubated at 32°C for 2 h with: glucose + yeast extract □ —□ —□ ; glucose □ —□ —□ .)



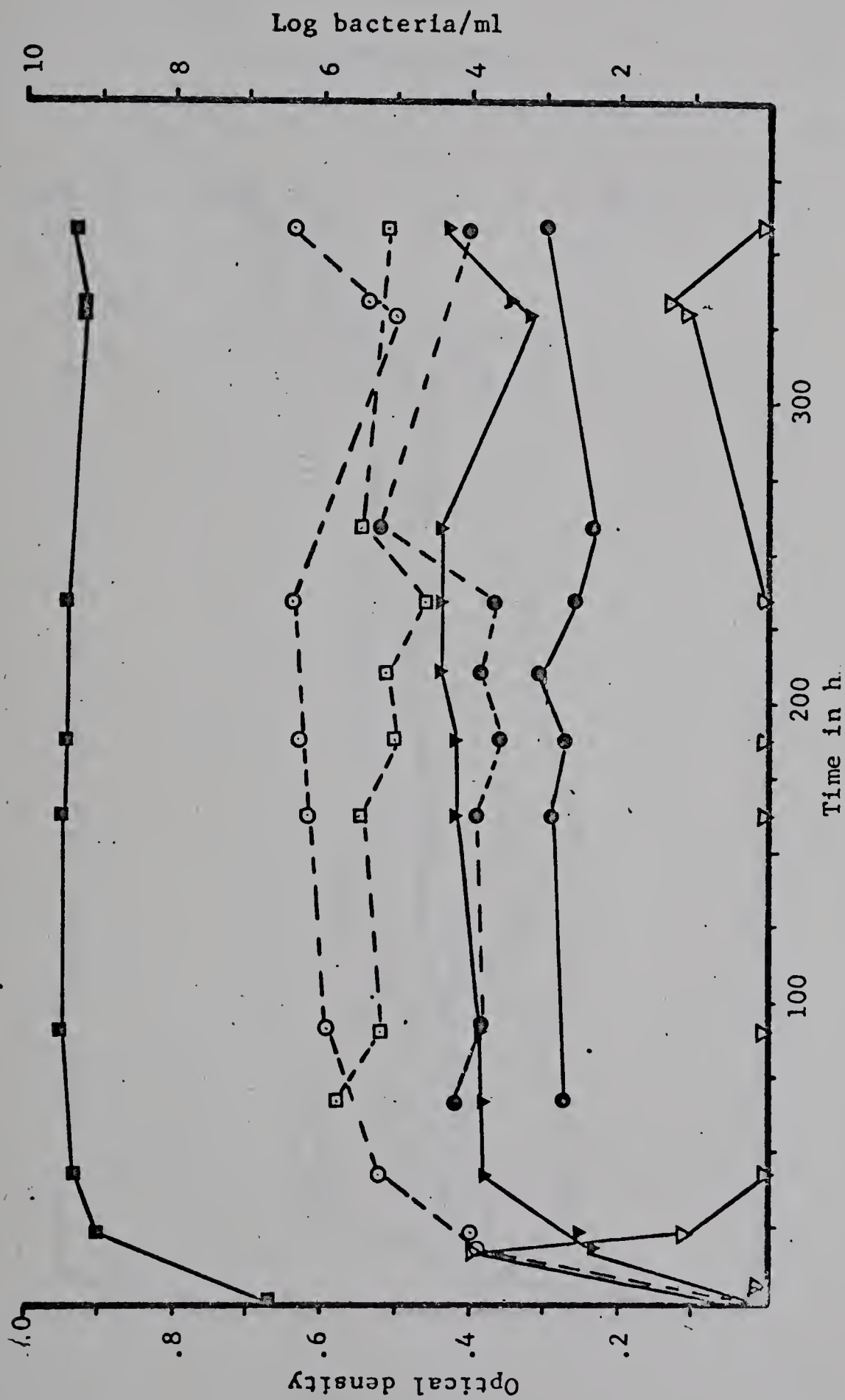


Fig. 13. Growth of *E. coli* in continuous culture with minimal medium. (See Appendix, Table 11). (The dilution rate was 0.1/h and the temperature was maintained at 32°C. Log bacteria/ml ■; turbidity of the culture ▼; dehydrogenase activity at 32°C for 20 min of a 10 ml sample from the chemostat with: water ▼; glucose O---O; dehydrogenase activity of the 1:10 dilution of the washed bacterial suspension with the turbidity adjusted to OD 1.040 and incubated at 32°C for 2 h with glucose □---□; malic acid ●---●; Na lactate ●---●.)



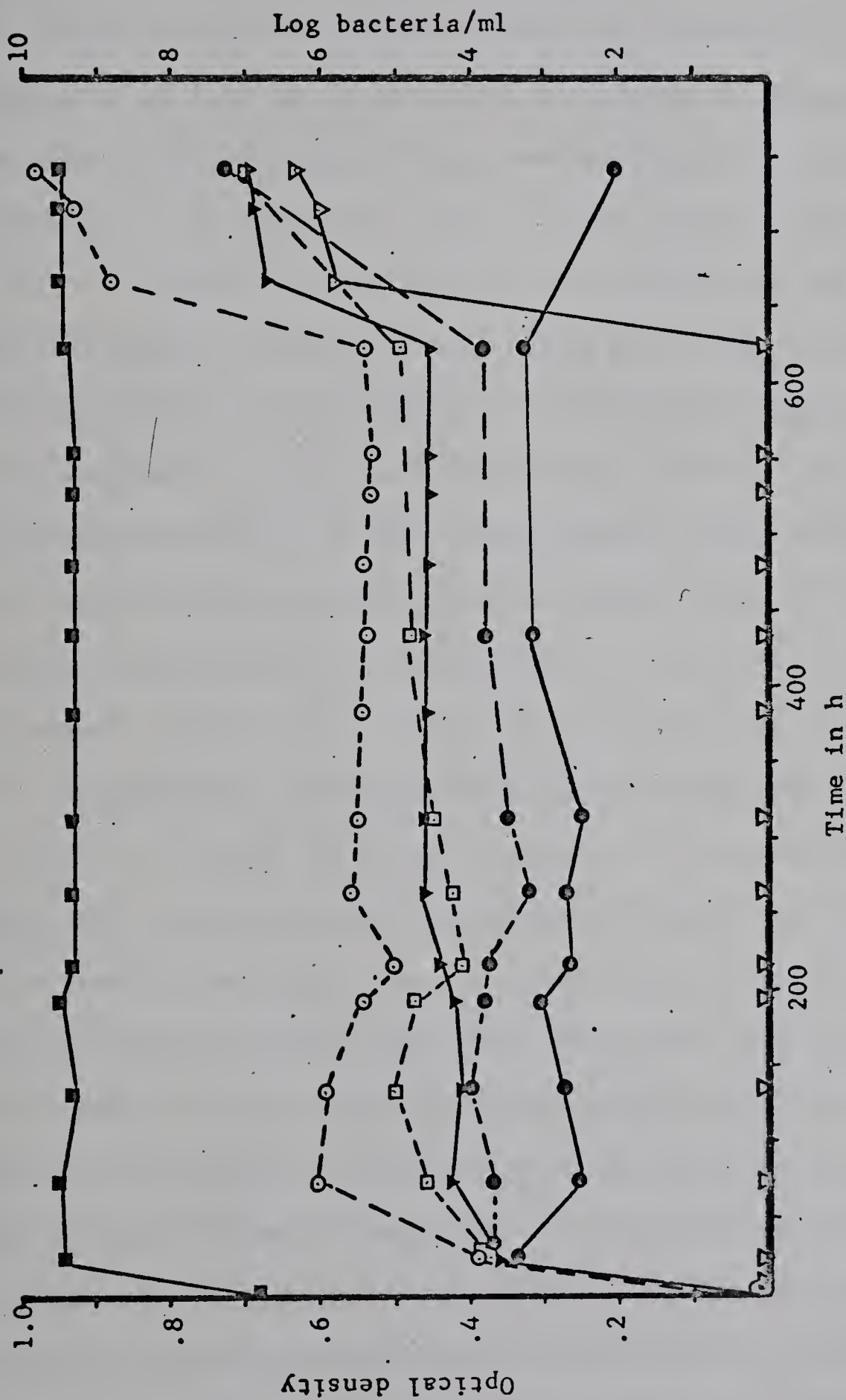


Fig. 14. Growth of *E. coli* in continuous culture with minimal and complete medium. (See Appendix, Table 12).  
 (The dilution rate was 0.1/h and the temperature was maintained at 32°C; at 624 h the chemostat was switched to complete broth. Log bacteria/ml  $\blacksquare$  ; turbidity of the culture  $\blacktriangle$  ; dehydrogenase activity at 32°C for 20 min of a 10 ml sample from the chemostat with: water  $\nabla$  ; glucose  $\circ$  ; dehydrogenase activity of the 1:10 dilution of the washed bacterial suspension with the turbidity adjusted to OD 1.040 and incubated at 32°C for 2 h with: glucose  $\square$  ; malic acid  $\bullet$  ; Na lactate  $\bullet$  .)





of reduction responses after the chemostat was changed to complete broth (Fig. 12). However, it would seem from Figs. 13 and 14 that the reliability of the TTC test depends on exhaustion of utilizable carbohydrates. It can be seen that no formazan is formed when water was used as the substrate, except when oxidizable carbohydrates appear not to be exhausted. Fig. 14 shows strong formazan production of culture samples with water when the chemostat was fed with complete broth. Formazan is also formed when water was used as the substrate in Fig. 13 when the cell concentration dropped, as shown by a decrease in culture turbidity at 250 to 350 h. Less variation in formazan production is shown when washed E. coli samples were used instead of the samples withdrawn directly from the culture vessel; however, preadjustment of the turbidity of the washed bacterial suspension did not reflect the state of operation of the chemostat. It is interesting to note that incomplete utilization of the medium in Fig. 13 at around 250 to 350 h resulted in a depression of formazan production when glucose was used as the substrate when bacteria were taken from the chemostat. On the other hand more formazan was produced by the washed E. coli when lactate was used as the substrate. This suggests pile-up of intracellular lactic acid possibly due to insufficient aeration and the operation of a glucose-lactate antagonism which was noted several times later. It appeared from these experiments that formazan production could be used as a convenient quick method to monitor steady state operation or variation in cell concentration in continuous culture of E. coli. The use of pure substrates is



preferred to mixtures of glucose and yeast extract; however, it is important that all utilizable carbohydrates be exhausted completely if glucose is used as the substrate. The use of lactate may be advisable when insufficient aeration results in accumulation of lactic acid by the culture.

Whereas steady state operation probably did not occur in the runs recorded in Figs. 11 and 12, it can be clearly seen that variations in dehydrogenase activity are minimized in the runs recorded in Figs. 13 and 14. Culture samples from the above mentioned four runs were used to study the effect of hypochlorite on E. coli and whether the type of damage inflicted could be shown by dehydrogenase activity measurements.

#### THE INVOLVEMENT OF VARIOUS DEHYDROGENASES IN DEATH OF BACTERIA BY CHLORINE AS ILLUSTRATED BY TETRAZOLIUM SALT REDUCTION

##### The Use of Complex and Specific Substrates to Illustrate Metabolic Damage by Chlorine.

As Knox et al. (1948) had shown that chlorine kills bacteria by inactivating glucose oxidizing enzymes and other essential sulfhydryl enzymes, it was considered that the tetrazolium staining technique, detailed in the previous section, might be useful in studying the involvement of various dehydrogenases in the resistance of bacteria to chlorine. The work of Milbauer and Grossowicz (1959 a) suggested that enzymes involved in glucose utilization were very susceptible to chlorine inactivation, and the





same authors (Milbauer and Grossowicz, 1959 b) showed that yeast extract appeared to "induce" the production of enzymes or metabolites in E. coli thereby considerably enhancing chlorine-resistance. Early work by the author indicated that yeast extract contained large amounts of readily oxidizable metabolites resulting in a pronounced production of formazan from TTC when yeast extract was the only source of metabolites. In fact, it was found that yeast extract and glucose were the substrates responsible for most of the formazan produced by E. coli from TTC when complete broth was used as the substrate for vital staining. It was therefore decided to study the effect of chlorine on the ability of E. coli to oxidize glucose and a glucose-yeast extract mixture approaching concentrations of complete broth. A few experiments will be shown which included the casein digest concentrate; however, for the sake of clarity the following summary of a disinfectant experiment is repeated here:

The chlorine (sodium hypochlorite) was added to 90 ml of buffered (pH 7.2) demineralized dilution water at a concentration sufficiently high that the final chlorine concentration as stated in the results was obtained when 10 ml of the washed bacterial suspension, with a turbidity of OD 1.040 using a no. 42 filter, was added. Samples were withdrawn at stated intervals. One ml samples were added to 9 ml sodium thiosulfate blanks, and held at 0°C for plating, whereas 10 ml samples were withdrawn and added to 2 ml substrate in colorimeter tubes for estimating metabolic damage. The substrate-TTC-neutralizer mixture always contained



0.5 ml of 1% buffered sodium thiosulfate and 0.5 ml of a 4800 ppm buffered TTC solution to give a final TTC concentration of 200 ppm after addition of 10 ml of bacterial suspension. Other substrates were added in 0.5 ml amounts as indicated, and the volume adjusted to 2 ml with water or buffer as required. The substrate tubes were kept in the 32°C waterbath and returned immediately after addition and mixing of the 10 ml bacterial sample. Incubation was for 2 h, unless otherwise specified, and the temperature used for the disinfectant exposure was 32°C or 20°C as indicated.

Frequently the sodium thiosulfate titration was performed on the 100 ml bacteria-chlorine mixture after 15 or 30 sec reaction time. The absence of detectable amounts of available chlorine suggested that the 10 ml of bacterial suspension caused rapid depletion of chlorine. This can also be concluded from survivor curves, which usually show a rapid decrease of bacteria in the first 15 sec of contact time. Small decreases which occurred after the initial rapid decrease in survivors are probably due to the sustained action of chloramines formed initially.

The use of complex substrates and glucose to illustrate enzymic damage by chlorine.

Fig. 15 shows that the time of incubation at 32°C is not an important factor in illustrating dehydrogenase damage by chlorine. Fig. 15 shows that graphs obtained after 1 and 2 h incubation are essentially parallel. Since 2 h incubation allowed sufficient time





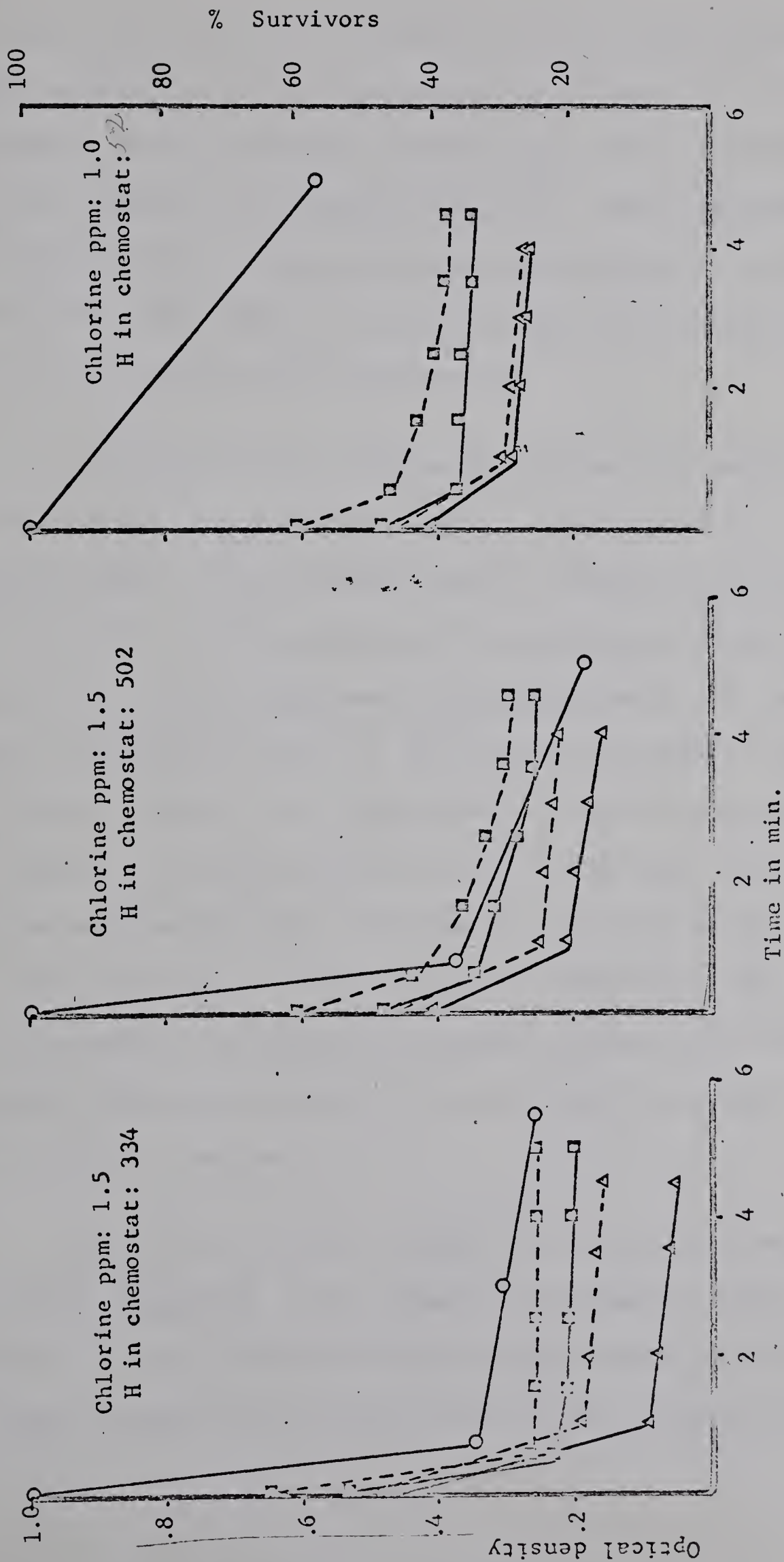


Fig. 15. The effect of incubation time for 60 and 120 min in illustrating dehydrogenase damage of *E. coli* grown in the chemostat in minimal medium (See Fig. 11). *E. coli* exposed to chlorine at 32°C. (Data recorded in Table 13 of the Appendix).  
(Percent survivors ○—○ ; dehydrogenase response to glucose + yeast extract incubated for 60 min, ■—■ ; 120 min ■---■ ; dehydrogenase response to glucose incubated for 60 min △—△ ; 120 min △---△ .)





for making plate counts for bacterial survival before optical density readings in the colorimeter had to be taken, 2 h incubation was adopted in all experiments, except those done on bacteria from the first chemostat run recorded in Fig. 11. These experiments are graphed in Fig. 15 and additional supporting data are recorded in Table 15 of the Appendix, in order to show that the good agreement in Fig. 15 was a general phenomenon.

Fig. 16 indicates differences obtained when washed bacteria from the run recorded in Fig. 11 were exposed to 1.5 and 2.0 ppm chlorine. The incubation time for formazan production of bacteria was 2 h. This constituted the experimental design for studying the chlorine resistance of E. coli grown in the chemostat in the run recorded in Fig. 12. The results are graphed in Figs. 17a, b and c. There is one unexplainable deviation when bacteria were exposed to 1.5 ppm after 168 h in the chemostat. This appears to be an experimental error since the 2.0 ppm curve agrees well with other experiments. There is quite a difference in the curves when the chemostat was switched to complete medium (Fig. 17c) suggesting a greater resistance of the individual bacterial cells from the complete medium.

Fig. 18 represents an example from a number of experiments done with E. coli grown in the chemostat and complete broth batch cultures. It can be noted that dehydrogenase damage by chlorine (1.0 ppm) is shown with all substrates used when bacteria were



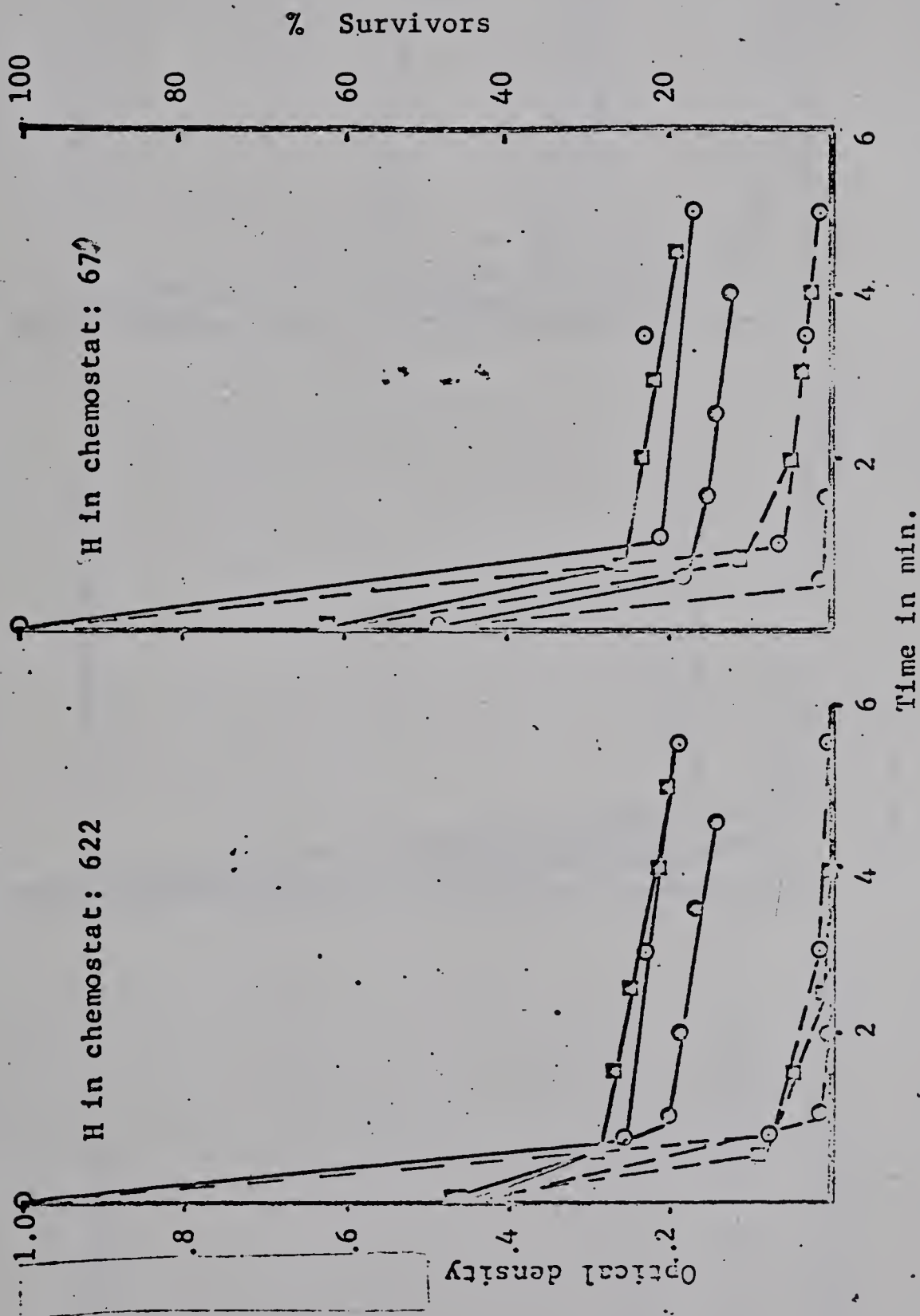


Fig. 16. Metabolic damage and death of *E. coli* on exposure to 1.5 and 2.0 ppm chlorine at 32°C and pH 7.2. *E. coli* grown in the chemostat in minimal medium. (See Fig. 11). (Data recorded in Table 14 of the Appendix.) (Exposure to chlorine at 1.5 ppm ———; 2 ppm -----; % survivors O-----O; dehydrogenase response to glucose + yeast extract O-----O; and glucose O-----O substrate.)





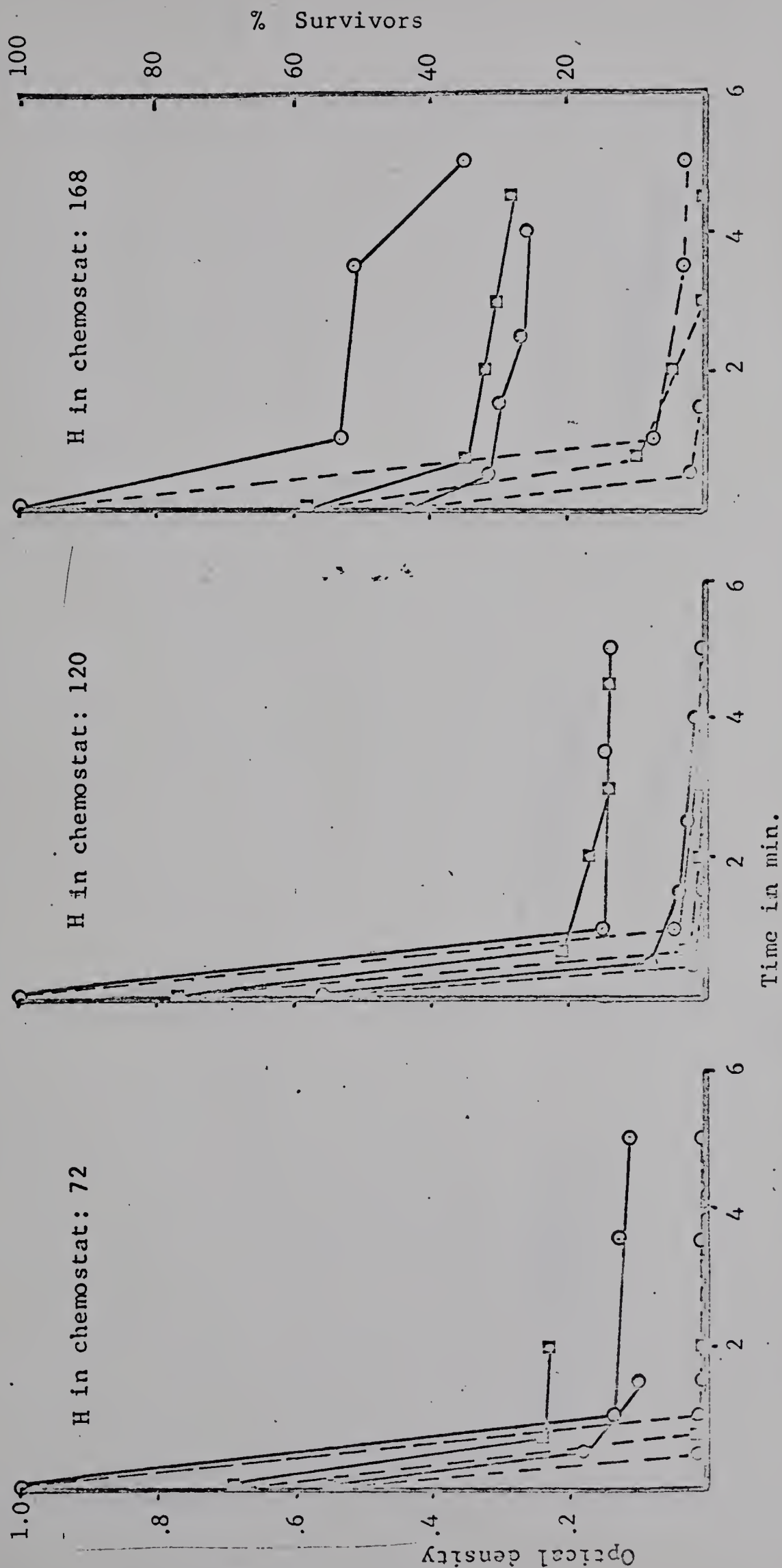


Fig. 17a. Metabolic damage and death of *E. coli* on exposure to 1.5 and 2.0 ppm chlorine at pH 7.2 and 32°C. *E. coli* grown in chemostat in minimal medium. (See Fig. 12). (Data recorded in Table 17 of the Appendix.) (Exposure to chlorine at 1.5 ppm ———; 2 ppm -----; % survivors O-----O; dehydrogenase response to glucose + yeast extract ■-----■; and glucose ●-----● substrate.)



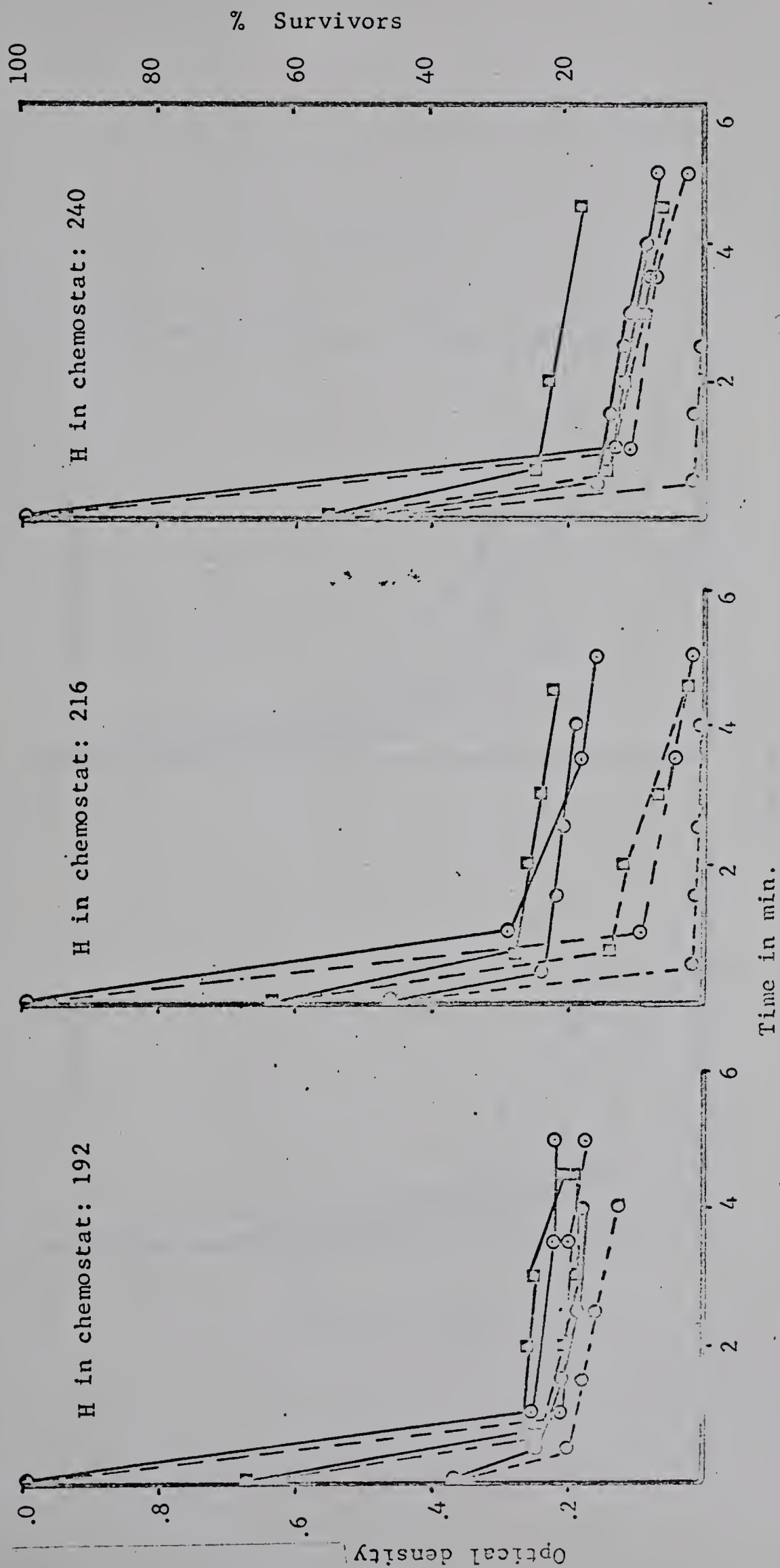


Fig. 17b. Metabolic damage and death of *E. coli* on exposure to 1.5 and 2.0 ppm chlorine at pH 7.2 and 32°C. *E. coli* grown in chemostat in minimal medium. (See Fig. 12). (Data recorded in Table 17 of the Appendix.) (Exposure to chlorine at 1.5 ppm —; 2 ppm ----; % survivors O---O; dehydrogenase response to glucose + yeast extract ■-----■; and glucose ●-----● substrate.)



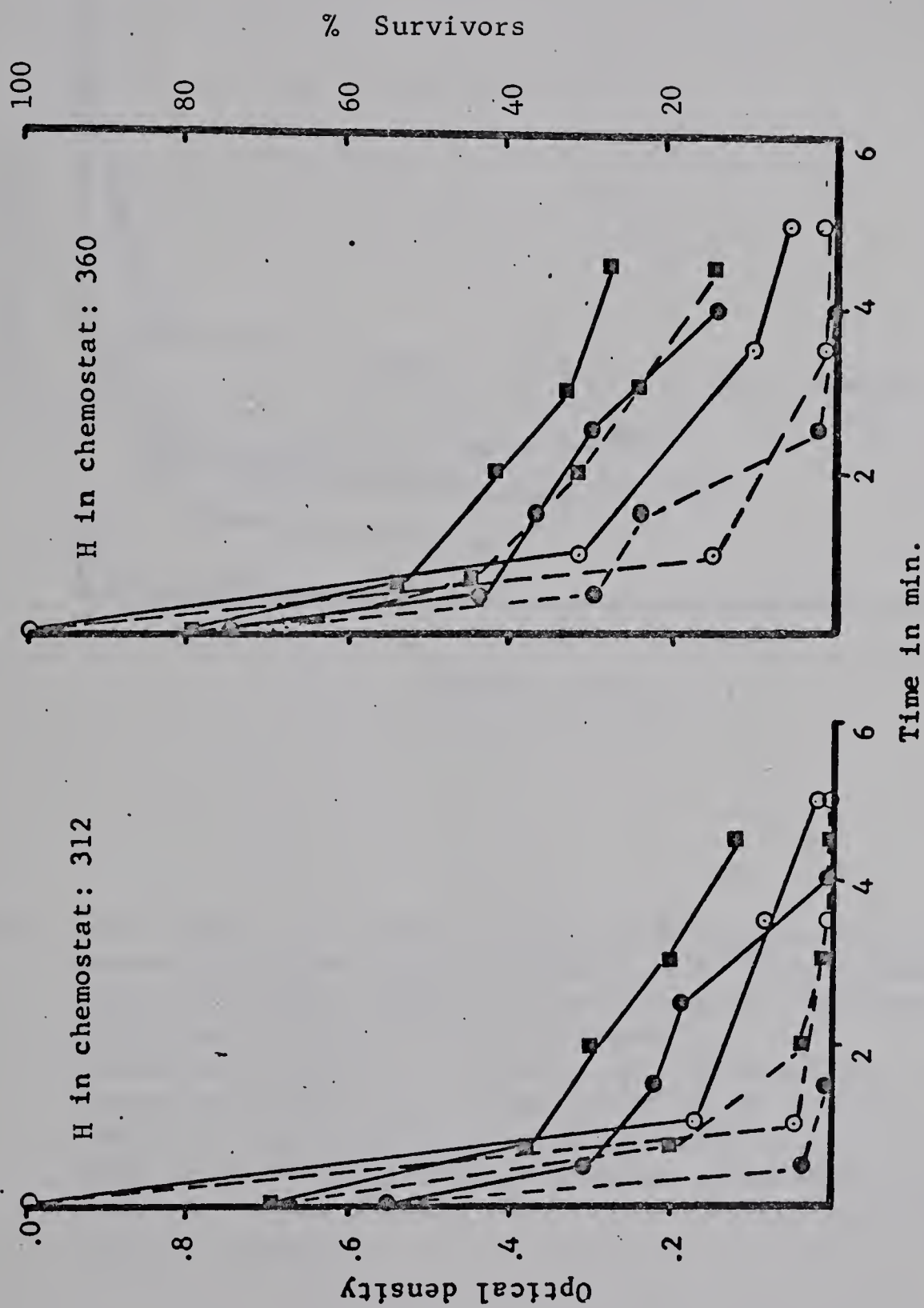


Fig. 17c. Metabolic damage and death of *E. coli* on exposure to 1.5 and 2.0 ppm chlorine at pH 7.2 and 32°C. *E. coli* grown in chemostat fed with complete broth. (See Fig. 12). (Data recorded in Table 17 of the Appendix.) (Exposure to chlorine at 1.5 ppm —; 2 ppm —; % survivors O---O ; dehydrogenase response to glucose + yeast extract ■-----■ ; and glucose ●-----● substrate.)





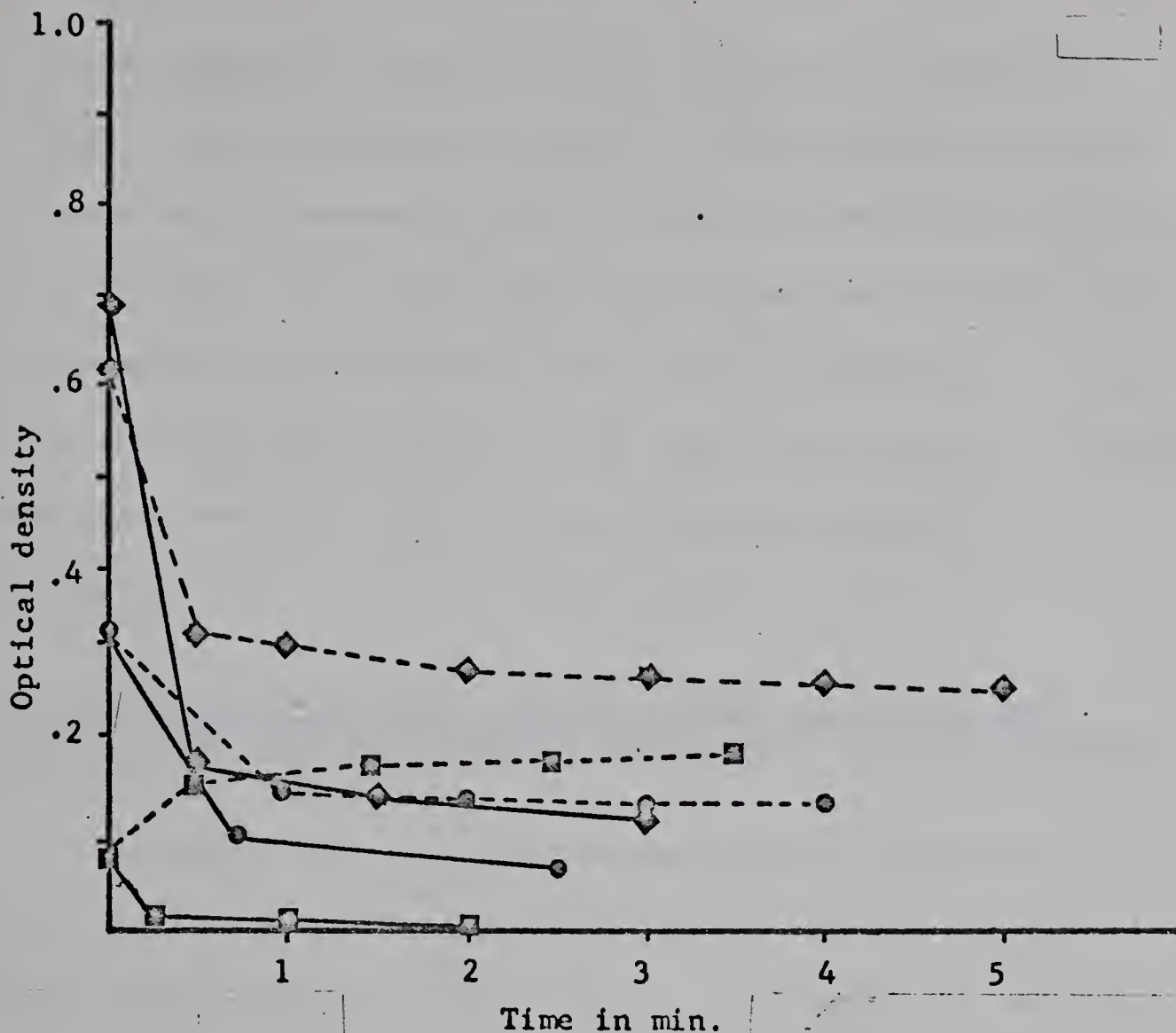


Fig. 18. An example of metabolic damage of *E. coli* with varying growth histories caused by exposure to 1 ppm chlorine. (Data and other examples illustrating the same phenomenon are given in Table 16 of the Appendix.) Chemostat culture (1) grown in minimal broth is indicated by solid lines, — ; batch culture (3) grown in complete broth is indicated with broken lines, -----. Substrates are indicated as follows: Complete broth  $\diamond$ ----- $\diamond$  ; yeast extract plus casein digest,  $\bullet$ ----- $\bullet$  ; glucose,  $\blacksquare$ ----- $\blacksquare$  .



grown in the chemostat; however, with E. coli grown in complete broth batch culture an apparent increase in dehydrogenase activity can be noted after exposure to chlorine when glucose is the substrate in the tetrazolium test. Data from repeat experiments showing the same phenomenon are presented in Table 16 of the Appendix. It was felt that this peculiar behaviour of E. coli after exposure to chlorine involved inactivation of certain highly reactive enzymes as will be shown later.

The use of specific substrates (glucose, malic acid and sodium lactate) to illustrate dehydrogenase damage of E. coli by chlorine.

The use of glucose substrate and complex substrates clearly indicated that other dehydrogenases besides glucose dehydrogenase were susceptible to chlorine inactivation. Attempts were therefore made to use pure substrates to illustrate damage of specific loci. It was noted that sodium citrate did not result in formazan production from TTC, suggesting poor penetration or crypticity of the enzyme locus involved. Good formazan production resulted when malic acid and sodium lactate were used as substrates.

Glucose, malic acid and sodium lactate were prepared in 1M solutions and used alone or in mixtures by adding 0.5 ml of a particular substrate solution. After addition of 10 ml of the bacterial suspension, the substrate concentration in the 12 ml reactant mixture was 1/24 M. Since it was desirable to study the decrease in specific dehydrogenases at the same exposure time,





chlorine action was stopped by adding 5 ml buffered sodium thiosulfate into the disinfectant suspension after a 1/2 min of exposure. When the 0.5 ml of neutralizer was not added to the substrate mixture in the tubes and 10.5 ml of the disinfectant treated bacterial suspension was added per tube, the 12 ml in the reactant tube was obtained as in other experiments where neutralizer was added to the colorimeter tubes and disinfectant action was arrested only after the 10 ml sample withdrawn from disinfection tube was mixed in the tube. This procedure was used to study the effect of chlorine on glucose, malic and lactic dehydrogenase in E. coli grown in minimal broth batch cultures as recorded in Table 18. It can be noted from this table that washed bacteria without additional substrate do not normally reduce TTC. This means that washing usually resulted in complete removal of available carbohydrates and is similar to the complete exhaustion of carbohydrates noted in the chemostat runs documented in Figs. 13 and 14. It could further be seen from the chemostat experiments that a culture which has no available carbohydrate will not reduce TTC (even when the tubes were incubated for 3h). When the sample withdrawn from the chemostat resulted in TTC reduction with water, as in Fig. 13 at around 330 h, it can be seen from the culture turbidity graph that steady state operation did not occur. Glucose dehydrogenase activity of the culture sample was depressed; however, lactic dehydrogenase activity of the washed bacterial suspension was increased. This glucose-lactic acid interaction on TTC reduction can also be observed in all cultures (Table 18); however, only the first experiment showed formazan production



Table 18. A study of the effect of chlorine on glucose, malic and lactic dehydrogenases of E. coli: (Cultures were grown in 1 l. minimal broth contained in 2 l. Erlenmeyer flasks with provision for aeration. After incubation at 32°C for 24 h a portion was used to prepare a suspension with a turbidity of OD 1.040. This suspension was used for disinfectant tests.)

| Culture history                          | Exposure to chlorine at 32°C |     | Optical density (formazan) with substrate concentration 1/24 M |         |            |                      |                      |                      |                     |                     | Count/ml | % survivors |
|------------------------------------------|------------------------------|-----|----------------------------------------------------------------|---------|------------|----------------------|----------------------|----------------------|---------------------|---------------------|----------|-------------|
|                                          |                              |     | Water                                                          | Glucose |            | Na lactate + glucose | Na lactate + glucose | Na lactate + glucose | Glucose + na malate |                     |          |             |
|                                          |                              |     |                                                                | malate  | Na lactate |                      |                      |                      |                     | Na lactate          |          |             |
| Not aerated, cell yield very low         | 0                            | 0   | .089                                                           | .305    | .245       | .550                 | .290                 | .388                 | .550                | 1.1x10 <sup>9</sup> | 100      |             |
|                                          | 1/2                          | 2.0 | 0                                                              | .012    | .010       | .090                 | .080                 | 0                    | 0                   | 1.6x10 <sup>8</sup> | 14.5     |             |
|                                          | 1/2                          | 1.5 | 0                                                              | .246    | .014       | .240                 | .260                 | .140                 | .200                | 4.4x10 <sup>8</sup> | 40.0     |             |
| Not aerated cell yield medium            | 0                            | 0   | 0                                                              | .510    | .220       | .580                 | .284                 | .464                 | .550                | 1.3x10 <sup>9</sup> | 100      |             |
|                                          | 1/2                          | 2.0 | 0                                                              | .144    | 0          | .130                 | .140                 | .040                 | .030                | 2.4x10 <sup>8</sup> | 18.4     |             |
|                                          | 1/2                          | 1.5 | 0                                                              | .240    | 0          | .174                 | .226                 | .130                 | .170                | 4.0x10 <sup>8</sup> | 30.8     |             |
| Vigorously aerated, cell yield medium    | 0                            | 0   | 0                                                              | .335    | .155       | .444                 | .138                 | .424                 | .460                | 1.3x10 <sup>9</sup> | 100      |             |
|                                          | 1/2                          | 2.0 | 0                                                              | .172    | 0          | .170                 | .190                 | .110                 | .110                | 5.7x10 <sup>8</sup> | 43.8     |             |
|                                          | 1/2                          | 1.5 | 0                                                              | .270    | 0          | .230                 | .280                 | .190                 | .230                | 6.4x10 <sup>8</sup> | 49.2     |             |
| Vigorously aerated, cell yield very high | 0                            | 0   | 0                                                              | .160    | .206       | .430                 | .070                 | .408                 | .270                | 1.3x10 <sup>9</sup> | 100      |             |
|                                          | 1/2                          | 2.0 | 0                                                              | 0       | 0          | 0                    | 0                    | 0                    | 0                   | 3.7x10 <sup>7</sup> | 2.8      |             |
|                                          | 1/2                          | 1.5 | 0                                                              | .150    | 0          | .130                 | .156                 | .064                 | .046                | 2.1x10 <sup>8</sup> | 16.2     |             |





with water. Since this culture was not aerated and showed a rather low cell concentration after 24 h incubation, it is difficult to decide whether the dehydrogenase activity was due to internal lactic acid or insufficient removal of nutrients by washing. It should be noted that formazan formation was depressed with all cultures in Table 18 when a glucose plus sodium lactate was used as the substrate; however, only when E. coli cultures were grown under heavy aeration did this combination of substrates result in an increase in TTC reduction after exposure to chlorine. When sodium lactate plus sodium malate or glucose plus sodium malate were used as substrate mixtures, it could be noted that formazan production was increased approximately as one would expect; however, these substrate combinations resulted in rapid decreases in formazan production after exposure of the cultures to chlorine. This suggested that glucose and malic dehydrogenase are readily inactivated by chlorine. Further it should be noted that dehydrogenase measurements indicated that the last culture in Table 18 was quite susceptible to chlorine. This was in fact borne out by survivor counts.

In Table 19 the above altered procedure where disinfectant action was arrested after 1/2 min was used to treat E. coli which were harvested from the chemostat run graphed in Fig. 13. It should be noted that glucose and malic dehydrogenase appear to be more susceptible to chlorine than lactic dehydrogenase regardless of original amount. The percent survivor column indicates good reproducibility in these tests.





Table 19. The involvement of glucose, malic and lactic dehydrogenase in death of E. coli by chlorine. E. coli grown in the chemostat. (See Fig. 13).

| H in chemostat | Exposure to chlorine at 32°C | Optical density (formazan) with substrate concentration 1/24 M |     |       |         |           | Count/ml | % survivors |                     |                      |
|----------------|------------------------------|----------------------------------------------------------------|-----|-------|---------|-----------|----------|-------------|---------------------|----------------------|
|                |                              | min                                                            | ppm | Water | Glucose | Na malate |          |             | Na lactate          | Glucose + Na lactate |
| 66             | 0                            | 0                                                              | 0   | 0     | .580    | .280      | .420     | .548        | 1.7x10 <sup>9</sup> | 100                  |
|                | 1/2                          | 2.0                                                            | 0   | 0     | 0       | 0         | .060     | .068        | 1.4x10 <sup>8</sup> | 8.2                  |
|                | 1/2                          | 1.5                                                            | 0   | 0     | .190    | .060      | .204     | .210        | 5.1x10 <sup>8</sup> | 30.0                 |
| 162            | 0                            | 0                                                              | 0   | 0     | .550    | .282      | .394     | -           | 1.6x10 <sup>9</sup> | 100                  |
|                | 1/2                          | 2.0                                                            | 0   | 0     | 0       | 0         | .089     | -           | 1.8x10 <sup>8</sup> | 11.3                 |
|                | 1/2                          | 1.5                                                            | 0   | 0     | .207    | .084      | .194     | -           | 4.2x10 <sup>8</sup> | 26.3                 |
| 186            | 0                            | 0                                                              | 0   | 0     | .500    | .270      | .350     | -           | 1.4x10 <sup>9</sup> | 100                  |
|                | 1/2                          | 2.0                                                            | 0   | 0     | 0       | 0         | .062     | -           | 9.5x10 <sup>7</sup> | 6.8                  |
|                | 1/2                          | 1.5                                                            | 0   | 0     | .137    | .026      | .154     | -           | 4.4x10 <sup>8</sup> | 31.4                 |
| 210            | 0                            | 0                                                              | 0   | 0     | .516    | .315      | .398     | -           | 1.2x10 <sup>9</sup> | 100                  |
|                | 1/2                          | 2.0                                                            | 0   | 0     | 0       | 0         | .080     | -           | 1.5x10 <sup>8</sup> | 12.5                 |
|                | 1/2                          | 1.5                                                            | 0   | 0     | .180    | .080      | .200     | -           | 3.7x10 <sup>8</sup> | 30.8                 |
| 234            | 0                            | 0                                                              | 0   | 0     | .460    | .258      | .360     | -           | 1.6x10 <sup>9</sup> | 100                  |
|                | 1/2                          | 2.0                                                            | 0   | 0     | 0       | 0         | .042     | -           | 1.2x10 <sup>8</sup> | 7.5                  |
|                | 1/2                          | 1.5                                                            | 0   | 0     | .077    | 0         | .090     | -           | 4.0x10 <sup>8</sup> | 25.0                 |



In Table 20 A. aerogenes was included in the study to see whether the malic acid locus is generally more susceptible to chlorine destruction than either the glucose or lactic acid loci. It appears from these experiments that this is so.

The effect of glucose and lactate concentration on TTC reduction in mixtures of these substrates is studied in Table 21. It can be clearly seen that glucose concentration is the inhibiting factor. When only 1/100 the normal amount of glucose is added to the reaction mixture a pronounced increase in formazan formation is noted in both experiments. Sodium lactate concentration appears to have little or no effect on this phenomenon. It should also be noted that the positive response with the culture of E. coli with water alone in the first experiment resulted in the suppression of reduction in the presence of glucose, suggesting that the original response might have been caused by extra or intracellular lactic acid.

The effect of chloramine-T on E. coli grown in the chemostat.

Fig. 19 illustrates that death of E. Coli by chloramine-T is accompanied by inactivation of some dehydrogenases. Again it can be seen that glucose and malic dehydrogenase are rapidly inactivated; however, lactic dehydrogenase shows a slower reaction in the presence of 20, 30 and 50 ppm chlorine as chloramine-T. It is also apparent that chloramine-T might have been a better system to study the whole problem of kill of E. coli by disinfectants because of its slower rate of reaction.







Table 20. The involvement of glucose, malic and lactic dehydrogenase in death of E. coli and A. aerogenes grown in batch cultures and exposed to 2.0 ppm chlorine. (F indicates that flaky ppt was formed.)

| Culture history                                         | Exposure to chlorine at 20°C | Optical density (formazan) with substrate concentration 1/24 M |     |                      |           |            | Count/ml EMB agar 48 h 32°C | % survivors       |      |
|---------------------------------------------------------|------------------------------|----------------------------------------------------------------|-----|----------------------|-----------|------------|-----------------------------|-------------------|------|
|                                                         |                              | Water                                                          |     | Glucose + Na lactate |           |            |                             |                   |      |
|                                                         |                              | min                                                            | ppm | Glucose              | Na malate | Na lactate |                             |                   |      |
| <u>E. coli</u> grown in minimal broth at 32°C for 24 h  | 0                            | 0                                                              | 0   | .330                 | .330      | .620       | .215                        | $7.3 \times 10^8$ | 100  |
|                                                         | 1/4                          | 2.0                                                            | -   | -                    | -         | -          | -                           | $2.2 \times 10^7$ | 3.01 |
|                                                         | 1/2                          | 2.0                                                            | 0   | 0                    | 0         | .040       | .045                        | $2.0 \times 10^7$ | 2.7  |
| <u>E. coli</u> grown in complete broth at 32°C for 44 h | 0                            | 0                                                              | 0   | .185                 | .080      | .320       | .120                        | $3.6 \times 10^8$ | 100  |
|                                                         | 1/4                          | 2.0                                                            | -   | -                    | -         | -          | -                           | $1.5 \times 10^8$ | 41.7 |
|                                                         | 1/2                          | 2.0                                                            | 0   | .060                 | 0         | .100       | .125                        | $1.4 \times 10^8$ | 38.9 |
| <u>A. aerogenes</u> in minimal broth at 32°C for 24 h   | 0                            | 0                                                              | 0   | .060F                | .350      | .340F      | 0                           | $7.0 \times 10^8$ | 100  |
|                                                         | 1/4                          | 2.0                                                            | -   | -                    | -         | -          | -                           | $8.0 \times 10^7$ | 11.4 |
|                                                         | 1/2                          | 2.0                                                            | 0   | .080                 | 0         | .125       | .160                        | $5.2 \times 10^7$ | 7.4  |
| <u>A. aerogenes</u> in minimal broth at 32°C for 44 h   | 0                            | 0                                                              | 0   | .175F                | .280      | .300       | .034F                       | $7.7 \times 10^8$ | 100  |
|                                                         | 1/4                          | 2.0                                                            | -   | -                    | -         | -          | -                           | $4.0 \times 10^5$ | 0.05 |
|                                                         | 1/2                          | 2.0                                                            | 0   | 0                    | 0         | 0          | 0                           | $2.0 \times 10^5$ | 0.03 |
| <u>A. aerogenes</u> in minimal broth at 32°C for 24 h   | 0                            | 0                                                              | 0   | .130F                | .380      | .325F      | .075F                       | $4.3 \times 10^8$ | 100  |
|                                                         | 1/4                          | 2.0                                                            | -   | -                    | -         | -          | -                           | $4.0 \times 10^7$ | 9.3  |
|                                                         | 1/2                          | 2.0                                                            | 0   | 0                    | 0         | .020       | .010                        | $3.0 \times 10^7$ | 7.0  |



Table 21. A study of the influence of glucose and sodium lactate concentrations in substrate mixtures on the inhibition of dehydrogenase activity of E. coli. (Bacteria exposed to 2.0 ppm chlorine at 20°C and grown in batch cultures.)

| Culture history                                                   | Exposure to chlorine at 20°C | Optical density (formazan) with substrate concentration 1/24 M |      |         |           |            |                      |                  |                      |                       |      | Count/ml EMB agar 48 h 32°C | % survivors |
|-------------------------------------------------------------------|------------------------------|----------------------------------------------------------------|------|---------|-----------|------------|----------------------|------------------|----------------------|-----------------------|------|-----------------------------|-------------|
|                                                                   |                              | Water                                                          |      | Glucose | Na malate | Na lactate | Glucose + Na lactate | Gluc/100 lactate | Glucose + Na lact/10 | Glucose + Na lact/100 |      |                             |             |
|                                                                   |                              | min                                                            | ppm  |         |           |            |                      |                  |                      |                       |      |                             |             |
| <u>E. coli</u> grown in complete broth at 32°C for 24 h (stirred) | 0                            | 0                                                              | .205 | .060    | .170      | .450       | .105                 | .075             | .500                 | .075                  | .055 | 3.8x10 <sup>8</sup>         | 100         |
|                                                                   | 1/4                          | 2.0                                                            | -    | -       | -         | -          | -                    | -                | -                    | -                     | -    | 1.3x10 <sup>8</sup>         | 34.2        |
|                                                                   | 1/2                          | 2.0                                                            | 0    | .230    | 0         | .200       | .245                 | .230             | .210                 | .225                  | .225 | 1.2x10 <sup>8</sup>         | 31.6        |
| <u>E. coli</u> grown in complete broth at 32°C for 44 h (stirred) | 0                            | 0                                                              | 0    | .185    | .080      | .320       | .120                 | .110             | .400                 | .150                  | .180 | 3.6x10 <sup>8</sup>         | 100         |
|                                                                   | 1/4                          | 2.0                                                            | -    | -       | -         | -          | -                    | -                | -                    | -                     | -    | 1.5x10 <sup>8</sup>         | 41.7        |
|                                                                   | 1/2                          | 2.0                                                            | 0    | .060    | 0         | .100       | .125                 | .110             | .120                 | .140                  | .070 | 1.4x10 <sup>8</sup>         | 38.9        |





H in chemostat: 192  
Chlorine ppm: 10

H in chemostat: 216  
Chlorine ppm: 20

H in chemostat: 216  
Chlorine ppm: 30

H in chemostat: 216  
Chlorine ppm: 50

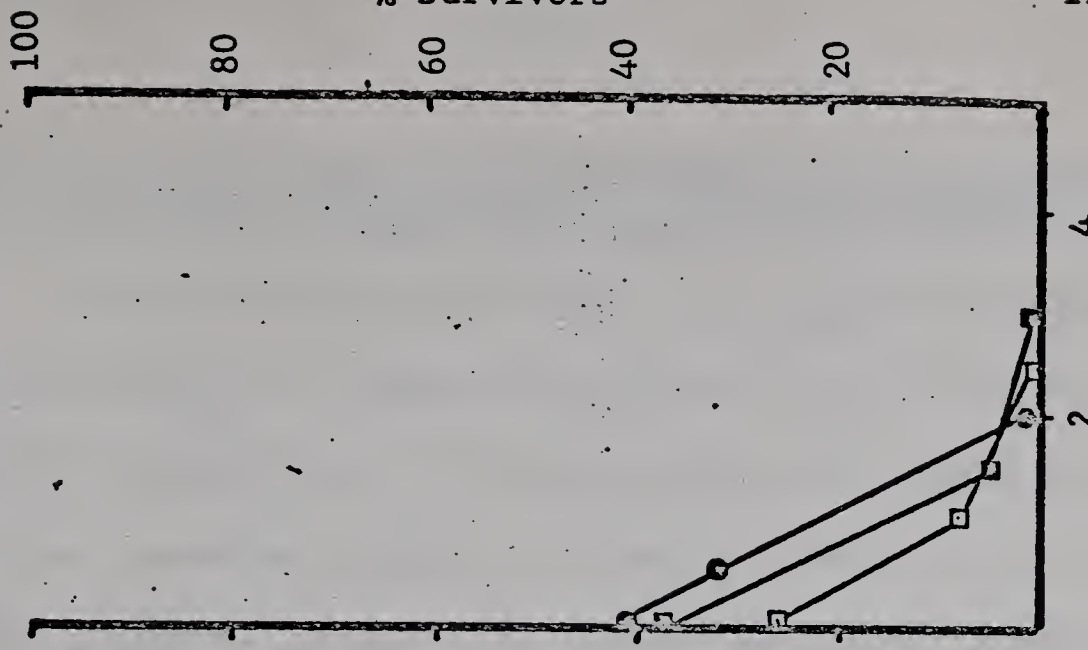
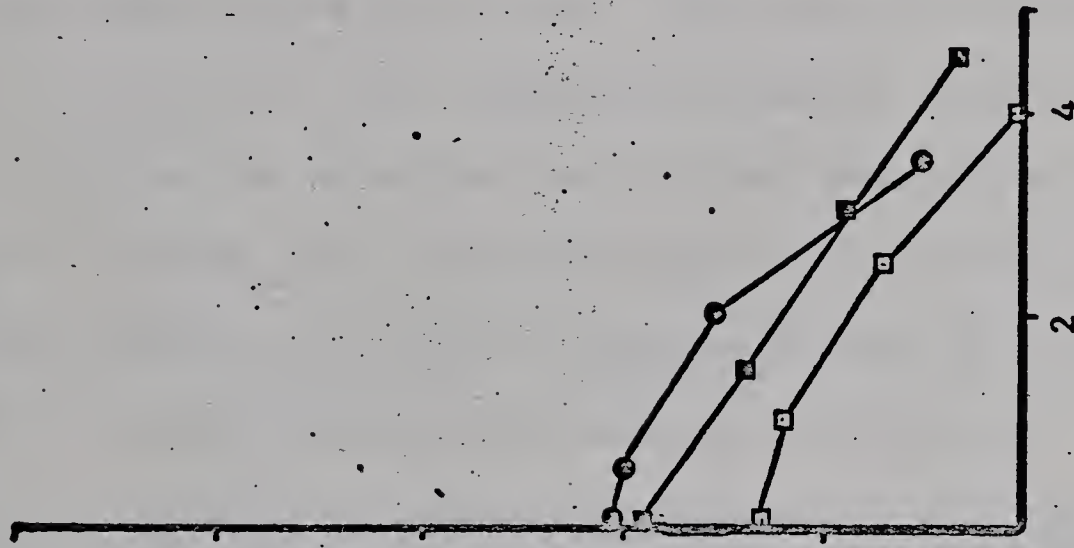
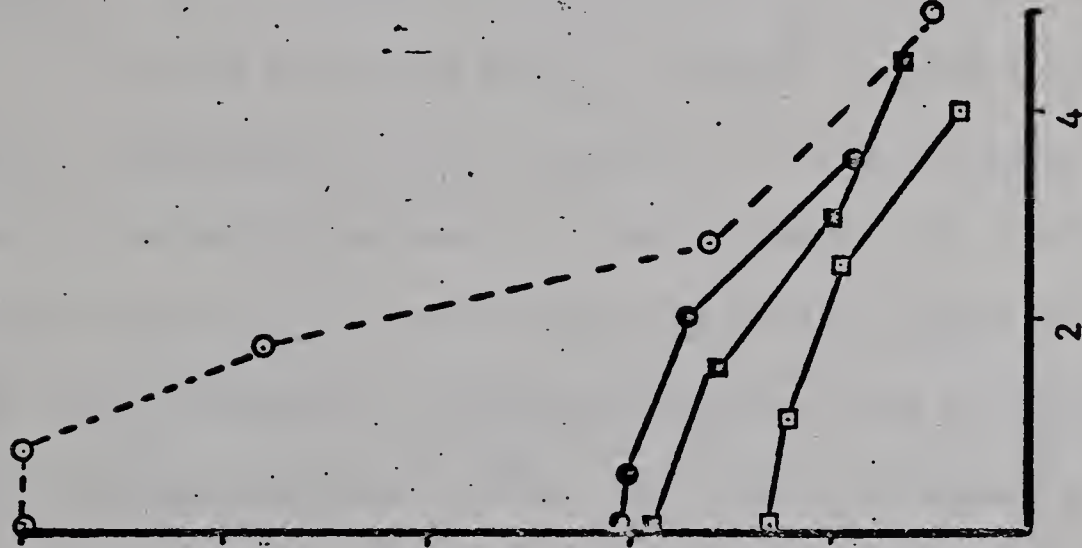
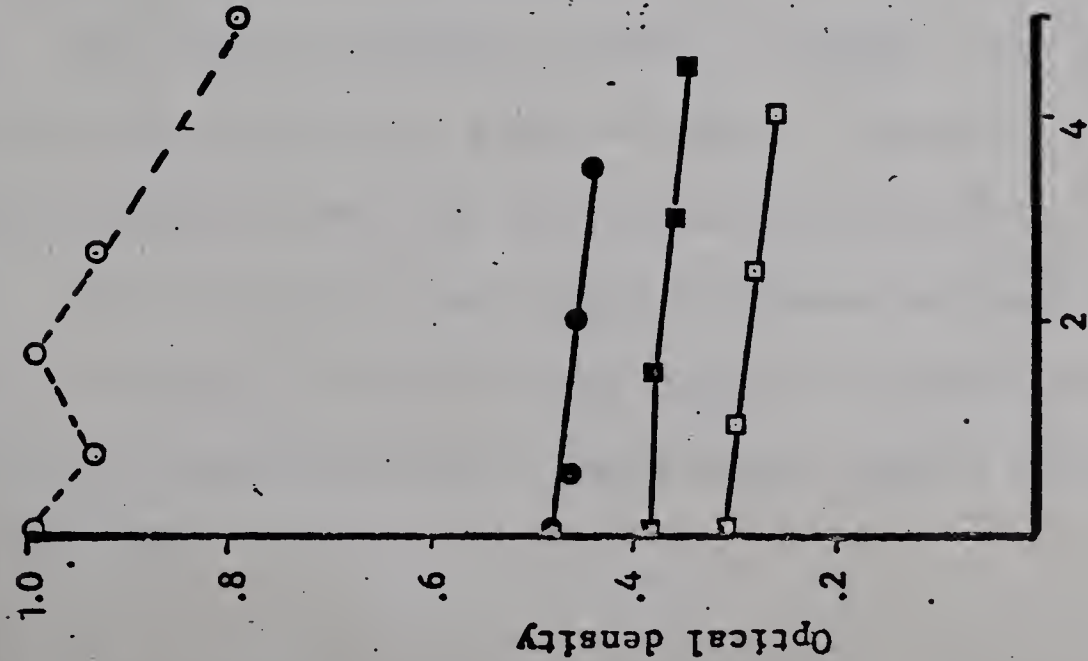


Fig. 19. The involvement of glucose, malic and lactic dehydrogenase in death of *E. coli* caused by chloramine-T at 20°C and pH 7.2. *E. coli* grown in the chemostat in minimal medium. (See Fig. 14).

(Dehydrogenase response to: glucose ●—●; na malate □—□; sodium lactate ■—■; % survivors O---O.)

(Data is recorded in Table 22 of the Appendix.)





Evidence of substrate protection as studied by pre-incubation of washed E. coli with a glucose prior to exposure to chlorine.

It had been noted that washed E. coli which reduced TTC without the addition of substrates appeared to be less susceptible to chlorine inactivation. It could be shown that mixing glucose and chlorine caused no reduction in available chlorine on titration and it was therefore thought that glucose may protect susceptible enzymes from inactivation by chlorine. This point is illustrated in Fig. 20 and Table 24. The compensations made in these experiments in preparing the substrates and chlorine solutions are described in the tables. The inhibitive effect on the lactic dehydrogenase on the addition of glucose is apparent as also is the reversal of the vital staining phenomenon on exposure to chlorine.

Evidence that variation in chlorine-resistance of E coli can be reduced when conditions in growing the bacteria are carefully controlled.

It can be seen from Fig. 12 and Fig. 14 that these two runs differ considerably in TTC-reduction of various samples. If TTC reduction by the dehydrogenases is a reflection of the physiological status of the cell the reduced variability would suggest that there should be less variation in chlorine-resistance from E. coli cultures harvested from the run shown in Fig. 14. Table 25 (Appendix) is therefore a compilation of all experiments done in that run using 2.0 ppm chlorine. When these are graphed in Fig. 21 together with experiments done with 2.0 ppm chlorine as shown in Table 17 (Appendix) it is apparent that bacteria grown in the last run show less variation in chlorine resistance. This is further proof of the importance of the physiological condition in chlorine-resistance of E. coli and indicates how greater reproducibility can be achieved in disinfectant testing than has been possible hitherto.



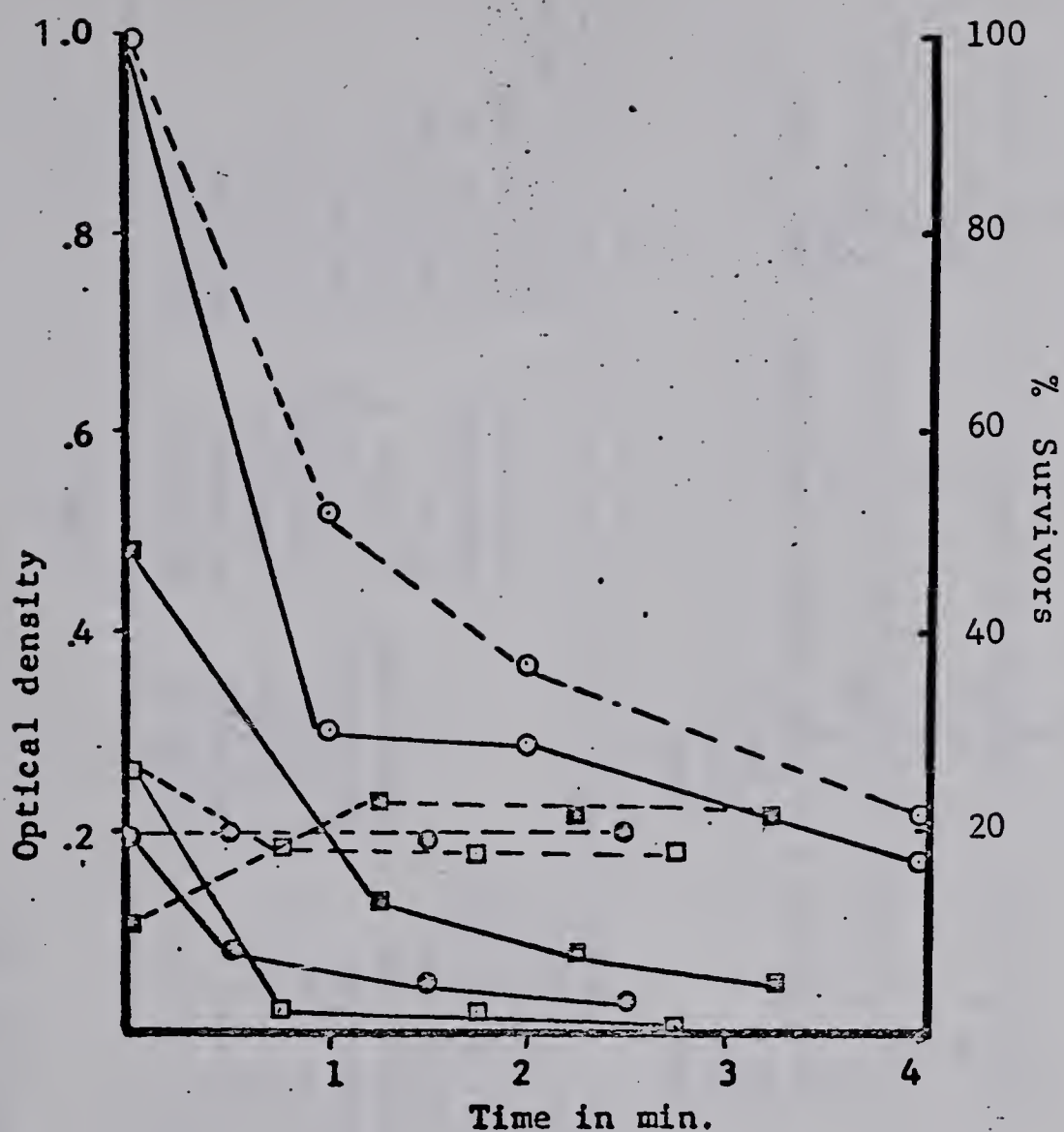


Fig. 20. Evidence that glucose may protect *E. coli* grown in minimal broth batch culture for 24 h at 32°C, from being killed by 2 ppm chlorine at 20°C and pH 7.2. (Data recorded in Table 23 of the Appendix.) (Washed culture preincubated with 1 ml of 1M glucose/10 ml suspension for 5 min at 20°C represented by ----- ; no glucose preincubation ——— ; dehydrogenase response to glucose ○-----○ ; na malate □-----□ ; sodium lactate ■-----■ ; % survivors indicated by ○-----○ .)





Table 24. Evidence that glucose may protect *E. coli* grown in the chemostat (minimal medium) from being killed by 2 ppm chlorine at 20°C and pH 7.2 (See Fig. 14).

| Exposure to chlorine at 20°C (min) | Substrates and counts                                      | Treatment of culture when grown in chemostat for:                                    |                                                         |                                                                                       |                                                                                                                  |
|------------------------------------|------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------------------------------------------|---------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------|
|                                    |                                                            | 24 h                                                                                 | 384 h                                                   |                                                                                       |                                                                                                                  |
| 0                                  | 90 ml of chlorine solution + 10 ml of bacterial suspension | 90 ml of chlorine solution containing 0.1 ml of glucose + 10 ml bacterial suspension | 90 ml of chlorine solution + 10 ml bacterial suspension | 90 ml of chlorine solution containing 1 ml of 1M glucose + 10 ml bacterial suspension | 89 ml of chlorine solution + 11 ml of bacterial suspension containing 1 ml of 1M glucose held at 20°C for 10 min |
| 0                                  | Water                                                      | 0                                                                                    | 0                                                       | 0                                                                                     | 0                                                                                                                |
| 0                                  | Glucose                                                    | .390                                                                                 | .390                                                    | .450                                                                                  | -                                                                                                                |
| 0                                  | Na malate                                                  | .340                                                                                 | .340                                                    | .250                                                                                  | -                                                                                                                |
| 0                                  | Na lactate                                                 | .380                                                                                 | .380                                                    | .350                                                                                  | -                                                                                                                |
| 0                                  | Count and %                                                | $1.4 \times 10^9$ (100)                                                              | $1.4 \times 10^9$ (100)                                 | $1.3 \times 10^9$ (100)                                                               | $1.3 \times 10^9$ (100)                                                                                          |
| 1/2                                | Glucose                                                    | 0                                                                                    | .090                                                    | 0                                                                                     | .030                                                                                                             |
| 3/4                                | Count and %                                                | $6.8 \times 10^7$ (4.9)                                                              | $2.0 \times 10^8$ (14.3)                                | $4.7 \times 10^7$ (3.6)                                                               | $2.0 \times 10^8$ (15.4)                                                                                         |
| 1                                  | Na malate                                                  | 0                                                                                    | 0                                                       | 0                                                                                     | 0                                                                                                                |
| 1 1/2                              | Na lactate                                                 | 0                                                                                    | .130                                                    | 0                                                                                     | .070                                                                                                             |
| 1 3/4                              | Count and %                                                | $3.7 \times 10^7$ (2.6)                                                              | $1.9 \times 10^8$ (13.6)                                | $4.1 \times 10^7$ (3.2)                                                               | $1.9 \times 10^8$ (14.6)                                                                                         |
| 2                                  | Glucose                                                    | 0                                                                                    | .070                                                    | 0                                                                                     | 0                                                                                                                |
| 2 1/2                              | Na malate                                                  | 0                                                                                    | 0                                                       | 0                                                                                     | 0                                                                                                                |
| 2 3/4                              | Count and %                                                | $5.7 \times 10^6$ (0.4)                                                              | $2.0 \times 10^8$ (14.3)                                | $3.8 \times 10^7$ (2.9)                                                               | 3.0                                                                                                              |
| 3                                  | Na lactate                                                 | 0                                                                                    | .090                                                    | 0                                                                                     | .050                                                                                                             |
| 3 1/2                              | Glucose                                                    | 0                                                                                    | .050                                                    | 0                                                                                     | 0                                                                                                                |
| 4                                  | Na malate                                                  | 0                                                                                    | 0                                                       | 0                                                                                     | 0                                                                                                                |
| 4 1/2                              | Na lactate                                                 | 0                                                                                    | .080                                                    | 0                                                                                     | .040                                                                                                             |
| 5                                  | Count and %                                                | $5.4 \times 10^6$ (0.4)                                                              | $2.0 \times 10^8$ (14.3)                                | $1.8 \times 10^7$ (1.4)                                                               | $1.7 \times 10^8$ (13.1)                                                                                         |



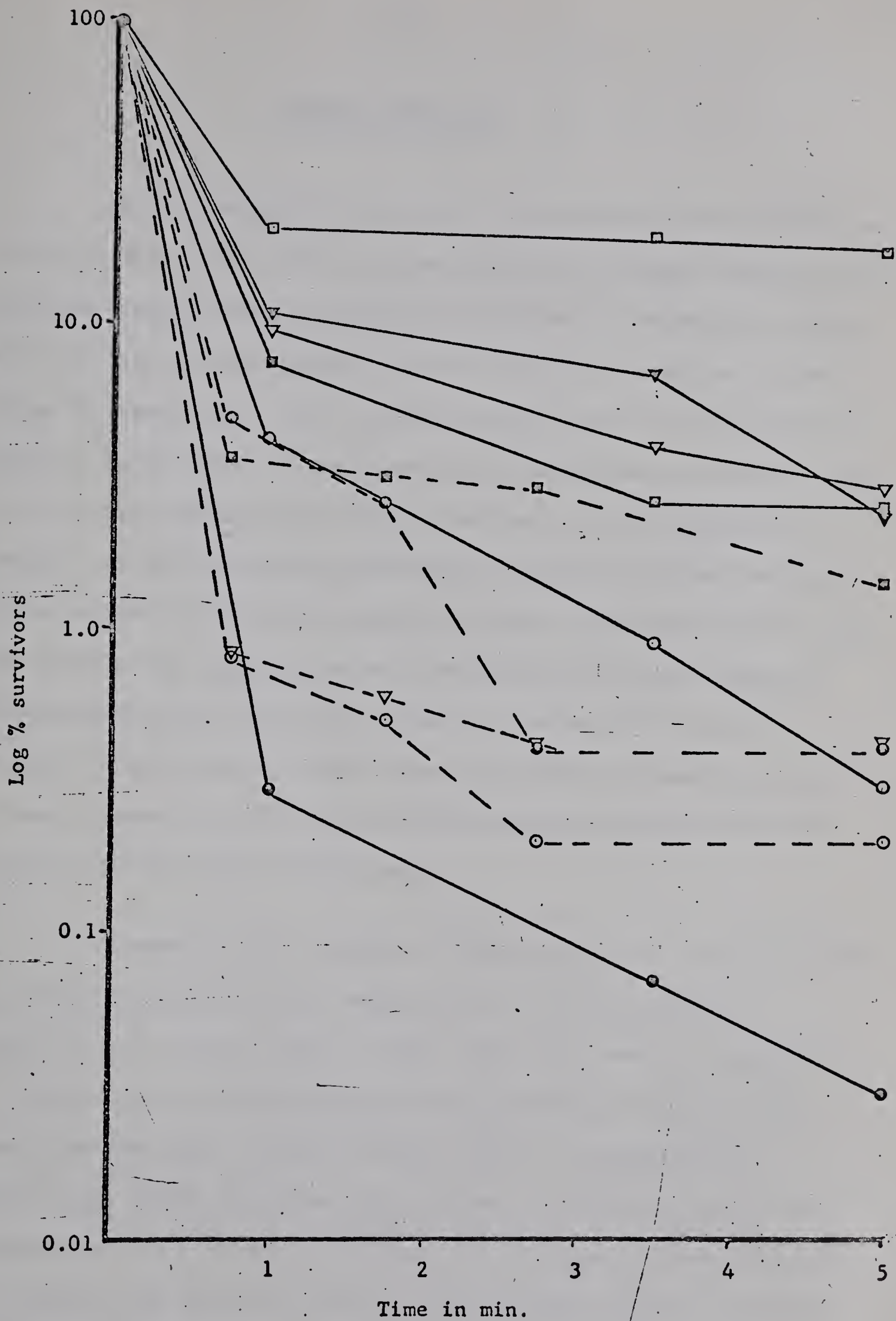


Fig. 21 Difference in chlorine-resistance of E. coli grown in two runs in the chemostat while operating with minimal broth. Washed E. coli suspensions were exposed to 2.0 ppm chlorine.

(Data represented by solid lines — are related to experiments with bacteria from the run recorded in Fig 12. H in chemostat are represented by:

72, ● ; 120, ○ ; 168, ■ ; 192, □ ; 216, ▽ ; 240, ▼ .

Data represented by dashed lines ---- are related to experiments with bacteria from the run recorded in Fig. 14. H in chemostat are represented by: 24, ● ; 72, ○ ; 384, ■ ; 432, ▽ . The Fig. represents all experiments done with 2.0 ppm while the chemostat was operated with minimal broth. Data recorded in Tables 17 and 25 of the Appendix.)







### GENERAL DISCUSSION

This investigation shows that a tetrazolium reduction test can be used to study some of the problems involved in changes which accompany bacterial death by chlorine bearing disinfectants; however, the lethal effect of TTC on dehydrogenases must be carefully considered in the design of experiments. Thus a study of publications in which TTC had been used to determine bacterial death by disinfectants, indicated that the final TTC concentration normally used was in the neighborhood of 500 ppm. It can be seen from experiments already discussed that such concentrations of TTC would markedly influence the sensitivity of tests when bacteria (E. coli) are grown in minimal broth in the chemostat. It was surprising to note that variation in susceptibility to TTC should be so great with a single strain of bacteria; however it was the establishment of this fact that made possible the use of the TTC test in the latter part of this work.

It appears that a number of dehydrogenase loci are associated with chlorine-resistance of E. coli and this may be regarded as support of the idea put forth by Eddy (1953) that death of bacteria by disinfectants involves progressive destruction of various independent enzyme sites. Further support for this is furnished by Gould et al. (1957) who showed that glucose, succinic and lactic dehydrogenases are involved in resistance of bacteria to hexachlorophene. The present work shows that certain dehydrogenases (glucose and malic) may be more susceptible to inactivation by chlorine than lactic



dehydrogenase. This basic information is necessary if progressive enzyme inactivation is used to explain differences in the shape of survivor curves observed in disinfection tests.

It was disappointing in the early experiments in this work to observe great variation in chlorine-resistance of E. coli grown in minimal broth in the chemostat. However, if variation in dehydrogenase results as found in samples withdrawn from the chemostat is a measure of overall physiological variation then this is not surprising. That such variations can occur in bacteria maintained in the chemostat has been supported by Wright and Lockhart (1965) who showed that the composition of E. coli grown under carefully controlled conditions was subject to change. This emphasizes the fact that changes in disinfectant resistance frequently observed on cultures with similar growth histories may in fact be caused by the great facility with which bacteria adapt themselves to slight changes in environment. Such differences may frequently be dismissed as resulting from the experimental method when survivor counts alone are used to illustrate damage inflicted on a bacterial culture by a disinfectant; however, it is more difficult to dismiss such differences when they are supported by simultaneous dehydrogenase determinations, as is the case in Figs. 17a, b and c. The changes in all measurements become especially apparent when the chemostat is changed to complete broth (See Fig. 17c). It is further shown in Fig. 21 that variation in chlorine-resistance can be reduced





when the chemostat is operated with extreme care.

An interesting phenomenon was the apparent increase in glucose dehydrogenase activity after chlorination of certain cultures as observed in Fig. 18 and Table 18. It is apparent from Table 21 that suppression of formazan formation can be achieved by mixing glucose and sodium lactate in the substrate; however, suppression of substrate oxidation appears to depend on glucose concentration only, since a marked increase in formazan formation is noted when only 1/100 the normal amount of glucose was added. Thus it appears that glucose causes a suppression of both glucose and lactic dehydrogenase. This suppression can be prevented by decreasing glucose concentration or perhaps glucose oxidation by inactivating glucose dehydrogenase by chlorine. This again furnishes evidence that of the three enzymes studied glucose dehydrogenase is preferentially inactivated by chlorine.

The effect of glucose, a carbohydrate which does not react with chlorine, in protecting E. coli from inactivation by that disinfectant as shown in Fig. 20 and Table 24 was rather surprising; however, Sadoff et al. (1965) have shown that glucose will protect glucose dehydrogenase extracted from bacterial spores from inactivation by a disinfectant. Protection of a pure enzyme extracted from spores, which usually show little metabolic activity, does not indicate that such a system may be important in reducing susceptibility of an actively metabolizing vegetative cell, but it is felt that the above observation may have wide spread application in survival of bacteria. Some experiments (not shown) indicated that glucose protection was dependent



on time. Thus no protection was noticed when bacteria were pre-incubated with glucose for less than 5 min at 20°C but protection appeared to reach a maximum at 15 - 20 min pre-incubation. Sodium malate appeared to behave similarly to glucose, whereas sodium lactate appeared to afford immediate protection which can be observed even when mixed with the disinfectant solution rather than the bacterial suspension, suggesting that protection by glucose against inactivation by chlorine is probably not because of protection of the glucose dehydrogenase locus alone, but pre-incubation with glucose allows conversion by the bacterial cell to lactate which in turn affords immediate protection against disinfection. This is illustrated to some extent in Table 24 where the greatest amount of formazan is produced by bacteria pre-incubated with glucose when sodium lactate is the major substrate. The fact that this increase is simply a result of the presence of glucose and sodium lactate can be disproved by showing that no TTC reduction is observed when glucose was mixed with the disinfectant solution.

In conclusion it can be stated that a decrease in the ability to utilize certain carbohydrates, i.e. glucose, malate and lactate can be illustrated by the TTC test when E. coli die due to the influence of chlorine. The fact that pre-incubation of E. coli with glucose tends to reduce the effectiveness of chlorine on the bacterial population suggests that some substrates may protect their specific enzymes against inactivation by chlorine. It may further be speculated that the inactivation of enzyme proteins may be of





major importance when death of bacteria was caused by chlorine since a decrease in death resulted from glucose protection. Thus the interaction of chlorine with competitive materials, such as proteins, or ammonia is not the only way in which the effectiveness of chlorine may be reduced, but also protection of susceptible enzymes may occur.

It would seem that a simple chemostat can not be used to produce reproducible bacterial inocula for disinfectant tests because of oscillation in the bacterial metabolic rate. However, the improvement brought about in reproducibility of disinfectant tests when the chemostat was operated with great care, i.e. frequent monitoring and manual adjustment of air and medium flow as well as continuous stirring of the medium supply to prevent stratification, suggests that a chemostat with automatic controls could be used to ensure a bacterial culture in the steady state which could be used to study the disinfectant action of chlorine.





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Table 1. The relationship between formazan production and bacterial concentration as illustrated by comparing turbidity of bacterial suspensions and optical density of solvent extracts of formazan. (See Fig. 3).

| % Bacterial suspension | Extraction of formazan with acetone and butanol |                            |                                         | Extraction of formazan with chloroform and <u>isopropynol</u> |                            |                                            |
|------------------------|-------------------------------------------------|----------------------------|-----------------------------------------|---------------------------------------------------------------|----------------------------|--------------------------------------------|
|                        | Optical density of 10 ml of suspension          | Calculated viable count/ml | Optical density of 5 ml butanol extract | Optical density of 10 ml of suspension                        | Calculated viable count/ml | Optical density of 5 ml chloroform extract |
| 100                    | 1.040                                           | $3.0 \times 10^9$          | 1.100                                   | 1.040                                                         | $3.22 \times 10^9$         | 1.060                                      |
| 75                     | .900                                            | $2.25 \times 10^9$         | -                                       | .900                                                          | $2.42 \times 10^9$         | .980                                       |
| 50                     | .730                                            | $1.5 \times 10^9$          | .770                                    | .760                                                          | $1.6 \times 10^9$          | .950                                       |
| 25                     | .480                                            | $0.75 \times 10^9$         | .268                                    | .492                                                          | $0.81 \times 10^9$         | .770                                       |
| 10                     | .234                                            | $0.30 \times 10^9$         | .240                                    | .254                                                          | $0.32 \times 10^9$         | .400                                       |
| 5                      | .134                                            | $0.15 \times 10^9$         | .048                                    | .134                                                          | $0.16 \times 10^9$         | .174                                       |
| 1                      | .024                                            | $0.03 \times 10^9$         | 0                                       | .034                                                          | $0.03 \times 10^9$         | .004                                       |
| 0 (blank)              | 0                                               | 0                          | 0                                       | 0                                                             | 0                          | 0                                          |

The final TTC concentration in the experiment extracted with acetone and butanol was 800 ppm, whereas 500 ppm TTC was used when formazan was extracted with chloroform and isopropynol. The colorimeter was equipped with a no. 42 filter, and bacterial suspensions were prepared from 24 h complete broth batch cultures.



Table 2. The effect of TTC concentration on vital staining of E. coli with different culture histories. (See Fig. 4).

| Bacterial culture | Incubation at 32°C | Optical density readings with the following concentration of TTC (ppm) |      |      |      |      |      |      | Viable count/ml   |
|-------------------|--------------------|------------------------------------------------------------------------|------|------|------|------|------|------|-------------------|
|                   |                    | Blank                                                                  | 100  | 200  | 400  | 600  | 800  | 1000 |                   |
| MB                | 10 min             | 0                                                                      | 0    | 0    | .004 | 0    | 0    | 0    | $1.8 \times 10^8$ |
|                   | 30 min             | 0                                                                      | 0    | 0    | 0    | 0    | 0    | 0    |                   |
|                   | 60 min             | 0                                                                      | 0    | .010 | .120 | .260 | .210 | .200 |                   |
|                   | 120 min            | 0                                                                      | 0    | .032 | .190 | .300 | .194 | .196 |                   |
| CB                | 10 min             | 0                                                                      | 0    | 0    | 0    | .006 | .006 | 0    | $7.7 \times 10^7$ |
|                   | 30 min             | 0                                                                      | 0    | 0    | .040 | .070 | .080 | .044 |                   |
|                   | 60 min             | 0                                                                      | 0    | .020 | .134 | .280 | .170 | .164 |                   |
|                   | 120 min            | 0                                                                      | .006 | .016 | .150 | .370 | .230 | .190 |                   |

The colorimeter was equipped with a no. 52 filter and washed bacterial suspensions of comparable turbidity were prepared from a 160 h minimal broth chemostat culture and a 24 h complete broth batch culture.



Table 3. The effect of TTC concentration on vital staining of E. coli maintained in a chemostat in minimal broth. (See Fig. 5).

| Incubation<br>at<br>32°C | Optical density readings with the<br>following concentration of TTC (ppm) |      |      |      |      |      |      | Viable<br>count/ml |
|--------------------------|---------------------------------------------------------------------------|------|------|------|------|------|------|--------------------|
|                          | Blank<br>0                                                                | 100  | 200  | 400  | 600  | 800  | 1000 |                    |
| 10 min                   | 0                                                                         | 0    | 0    | .006 | .010 | .012 | .012 |                    |
| 30 min                   | 0                                                                         | 0    | 0    | .010 | .010 | .012 | .012 | $7.4 \times 10^7$  |
| 60 min                   | 0                                                                         | 0    | 0    | .008 | .006 | .008 | .008 |                    |
| 10 min                   | 0                                                                         | .008 | .100 | .270 | .320 | .320 | .320 |                    |
| 30 min                   | 0                                                                         | .050 | .300 | .540 | .460 | .460 | .420 | $7.4 \times 10^8$  |
| 60 min                   | 0                                                                         | .082 | .390 | .620 | .520 | .540 | .500 |                    |

The colorimeter was equipped with a no. 52 filter and the bacterial suspension was prepared from a 190 h minimal broth chemostat culture.





Table 4. The effect of TTC concentration on vital staining of E. coli grown in complete broth in batch culture. (See Fig. 6).

| Incubation<br>at<br>32°C | Optical density readings with the<br>following concentration of TTC (ppm) |      |      |      |      |      |       |       | Viable<br>count/ml |
|--------------------------|---------------------------------------------------------------------------|------|------|------|------|------|-------|-------|--------------------|
|                          | Blank<br>0                                                                | 100  | 200  | 400  | 500  | 600  | 800   | 1000  |                    |
| 10 min                   | 0                                                                         | 0    | .016 | .062 | .140 | .256 | .514  | .620  | $1.8 \times 10^8$  |
| 30 min                   | 0                                                                         | .018 | .054 | .220 | .380 | .670 | .950  | 1.100 |                    |
| 60 min                   | 0                                                                         | .024 | .056 | .280 | .480 | .790 | 1.100 | 1.200 |                    |
| 90 min                   | 0                                                                         | .026 | .064 | .280 | .492 | .815 | 1.100 | 1.200 |                    |
| 10 min                   | 0                                                                         | 0    | 0    | .010 | .010 | .003 | .006  | 0     | $1.8 \times 10^7$  |
| 30 min                   | 0                                                                         | 0    | 0    | .004 | .007 | .007 | .004  | 0     |                    |
| 60 min                   | 0                                                                         | 0    | 0    | .004 | .006 | .007 | .006  | 0     |                    |
| 90 min                   | 0                                                                         | 0    | .016 | .120 | .117 | .010 | 0     | 0     |                    |

The colorimeter was equipped with a no. 52 filter and the bacterial suspension was prepared from a 24 h complete broth batch culture.



Table 5. The effect of TTC concentration and incubation time on vital staining of *E. coli* grown in batch culture in complete broth. (See Figs. 7a and b).

| Incubation<br>at<br>32°C | Optical density readings with the<br>following concentration of TTC (ppm) |      |      |      |      |      |      |       | Viable<br>count/ml  |
|--------------------------|---------------------------------------------------------------------------|------|------|------|------|------|------|-------|---------------------|
|                          | Blank<br>0                                                                | 100  | 200  | 400  | 500  | 600  | 800  | 1000  |                     |
| 15 min                   | 0                                                                         | 0    | .010 | .038 | .056 | .118 | .160 | .600  |                     |
| 30 min                   | 0                                                                         | 0    | .020 | .079 | .116 | .230 | .306 | .920  |                     |
| 60 min                   | 0                                                                         | 0    | .034 | .115 | .165 | .320 | .424 | .970  | 2.6x10 <sup>8</sup> |
| 90 min                   | 0                                                                         | 0    | .038 | .128 | .183 | .354 | .460 | .980  |                     |
| 120 min                  | 0                                                                         | .004 | .040 | .144 | .200 | .376 | .484 | 1.000 |                     |

The colorimeter was equipped with a no. 52 filter and the bacterial suspension was prepared from a 15 h complete broth batch culture.





Table 6. The effect of staining *E. coli* grown in complete broth in batch culture for 20 h. at 32°C in the presence of dead bacteria. (See Figs. 8a and b).

|                    |                                                       | Optical density readings with the<br>following concentration of TTC (ppm) |      |      |      |       |       |
|--------------------|-------------------------------------------------------|---------------------------------------------------------------------------|------|------|------|-------|-------|
|                    |                                                       | 300                                                                       |      | 500  |      | 700   |       |
| % Live<br>bacteria | Viable<br>count/ml<br>before addition<br>of substrate | Incubation at 32°C in min.                                                |      |      |      |       |       |
|                    |                                                       | 90                                                                        | 180  | 90   | 180  | 90    | 180   |
| 100                | 2.01x10 <sup>8</sup>                                  | .280                                                                      | .496 | .750 | .854 | 1.140 | 1.300 |
| 80                 | 1.61x10 <sup>8</sup>                                  |                                                                           |      | .730 | .854 |       |       |
| 60                 | 1.21x10 <sup>8</sup>                                  |                                                                           |      | .650 | .850 |       |       |
| 50                 | 1.00x10 <sup>8</sup>                                  |                                                                           |      | .580 | .800 |       |       |
| 40                 | 8.0x10 <sup>7</sup>                                   | .240                                                                      | .320 | .500 | .670 | .400  | .390  |
| 20                 | 4.0x10 <sup>7</sup>                                   | .160                                                                      | .220 | .280 | .290 | .240  | .240  |
| 10                 | 2.0x10 <sup>7</sup>                                   |                                                                           |      | .180 | .180 |       |       |
| 1                  | 2.0x10 <sup>6</sup>                                   |                                                                           |      | .030 | .080 |       |       |
| 0                  | 0                                                     |                                                                           |      | .014 | .016 |       |       |



Table 7. The effect of bacterial and TTC concentration and incubation time on vital staining of E. coli in the presence of dead bacteria. (See Figs. 9a and b).

| Optical density readings with the<br>following concentration of TTC (ppm) |                                           |                      |      |      |      |                              |      |      |      |       |      |      |       |       |
|---------------------------------------------------------------------------|-------------------------------------------|----------------------|------|------|------|------------------------------|------|------|------|-------|------|------|-------|-------|
| Viable<br>count/ml<br>before<br>addition of<br>substrate                  |                                           | 300                  |      |      | 400  |                              |      | 500  |      |       |      |      |       |       |
| % Live<br>bacteria                                                        | Turbidity<br>of 10 ml<br>suspension<br>OD | 1                    | 2    | 4    | 17   | Incubation time at 32°C in h |      |      |      | 1     | 2    | 4    | 17    |       |
|                                                                           |                                           |                      |      |      |      | 1                            | 2    | 4    | 17   |       |      |      |       |       |
| 100                                                                       | .240                                      | 2.4x10 <sup>8</sup>  | .210 | .280 | .520 | .950                         | .400 | .520 | .850 | 1.300 | .460 | .560 | .900  | 1.200 |
| 80                                                                        | .246                                      | 1.92x10 <sup>8</sup> |      |      |      |                              |      |      |      | .400  | .520 | .800 | 1.200 |       |
| 60                                                                        | .254                                      | 1.44x10 <sup>8</sup> |      |      |      |                              |      |      |      | -     | -    | -    | -     | -     |
| 40                                                                        | -                                         | -                    |      |      |      |                              |      |      |      | .350  | .460 | .700 | 1.000 |       |
| 20                                                                        | .256                                      | 4.8x10 <sup>7</sup>  |      |      |      |                              |      |      |      | .140  | .270 | .460 | .800  |       |
| 10                                                                        | .260                                      | 2.4x10 <sup>7</sup>  | .030 | .110 | .200 | .520                         | .040 | .180 | .300 | .650  | .040 | .200 | .340  | .700  |
| 5                                                                         | .260                                      | 1.2x10 <sup>7</sup>  |      |      |      |                              |      |      |      | .040  | .110 | .180 | .380  |       |
| 1                                                                         | .260                                      | 2.4x10 <sup>6</sup>  |      |      |      |                              |      |      |      | .040  | .050 | .110 | .280  |       |
| 0                                                                         | .254                                      | 0                    |      |      |      |                              |      |      |      | .030  | .040 | .040 | .160  |       |

A7

The culture of E. coli was grown in complete broth for 24 h at 32°C and the turbidity of the washed suspension was adjusted to OD 1.040 (no. 42 filter) before being diluted 1:10 for the test.









Table 8. The effect of bacterial and TTC concentration and incubation time on vital staining of *E. coli* in the presence of dead bacteria. (See Figs. 10a, b and c).

| % Live bacteria | Time at 32°C | Optical density readings with the following concentration of TTC (ppm) |                |      |                |      |      |      |      |
|-----------------|--------------|------------------------------------------------------------------------|----------------|------|----------------|------|------|------|------|
|                 |              | 0                                                                      | 100            | 200  | 300            | 400  | 500  | 600  | 700  |
| 100             | 0 h          | 0                                                                      | .002           | .002 | .014           | .018 | .006 | .010 | .016 |
|                 | 1 h          | .014                                                                   | .270           | .200 | .150           | .120 | .080 | .050 | .050 |
|                 | 2 h          | .034                                                                   | .190           | .200 | .150           | .120 | .080 | .054 | .050 |
|                 | 3 h          | .046                                                                   | .210           | .200 | .150           | .116 | .076 | .054 | .046 |
| 50              | 0 h          | .024                                                                   | .022           | .016 | .004           | .006 | .012 | .020 | .016 |
|                 | 1 h          | .016                                                                   | .082           | .100 | .072           | .058 | .036 | .020 | .016 |
|                 | 2 h          | .021                                                                   | .120           | .120 | .080           | .060 | .040 | .038 | .022 |
|                 | 3 h          | .046                                                                   | .134           | .126 | .080           | .060 | .050 | .040 | .034 |
| 10              | 0 h          | .006                                                                   | 0              | .016 | .004           | .012 | .001 | .014 | .010 |
|                 | 1 h          | 0                                                                      | 0              | .010 | .008           | .018 | .010 | .012 | .016 |
|                 | 2 h          | 0                                                                      | .030           | .044 | .030           | .020 | 0    | .004 | .006 |
|                 | 3 h          | .014                                                                   | (pink)<br>.044 | .054 | (pink)<br>.046 | .046 | .036 | .010 | .006 |
| 0               | 0 h          | .008                                                                   | .022           | .006 | .008           | .012 | .006 | .014 |      |
|                 | 1 h          | 0                                                                      | .008           | .010 | .008           | .010 | .006 | .014 |      |
|                 | 2 h          | 0                                                                      | 0              | 0    | 0              | 0    | 0    | .006 |      |
|                 | 3 h          | 0                                                                      | 0              | 0    | 0              | 0    | 0    | 0    |      |

The *E. coli* culture was maintained in the chemostat for 281 h with minimal broth at a dilution rate of 0.1/h at 32°C. After washing, turbidity of the suspension was adjusted to OD 1.040 before being diluted 1:10 for the test.



Table 9. Growth of E. coli in continuous culture. (See Fig. 11)

| Time<br>H        | pH  | Viable<br>count/ml   | Turbidity<br>OD | Relative dehydrogenase activity                        |         |                                                                       |         |
|------------------|-----|----------------------|-----------------|--------------------------------------------------------|---------|-----------------------------------------------------------------------|---------|
|                  |     |                      |                 | Sample from chemostat<br>Incubation: 20 min at<br>32°C |         | Washed bacteria <sup>†</sup> , OD<br>1.040 Incubation:<br>2 h at 32°C |         |
|                  |     |                      |                 | Glucose +<br>yeast extract                             | Glucose | Glucose +<br>yeast extract                                            | Glucose |
| 0                | 7.2 | 4.0x10 <sup>6</sup>  | 0               | 0                                                      | 0       | -                                                                     | -       |
| 70               | 6.8 | 6.8x10 <sup>8</sup>  | .240            | .450                                                   | .180    | -                                                                     | -       |
| 92               | 6.9 | 1.65x10 <sup>9</sup> | .380            | .770                                                   | .660    | .650                                                                  | .520    |
| 129              | 7.0 | -                    | .394            | -                                                      | -       | -                                                                     | -       |
| 142              | 7.0 | 1.10x10 <sup>9</sup> | .380            | .700                                                   | .600    | -                                                                     | -       |
| 149              | -   | -                    | .390            | -                                                      | -       | -                                                                     | -       |
| 239              | 7.2 | -                    | .360            | .420                                                   | .470    | -                                                                     | -       |
| 287              | 7.2 | 1.29x10 <sup>9</sup> | .320            | .510                                                   | .530    | -                                                                     | -       |
| 310              | -   | 1.04x10 <sup>9</sup> | .320            | .380                                                   | .410    | -                                                                     | -       |
| 334              | 7.1 | 1.45x10 <sup>9</sup> | .420            | .750                                                   | .750    | .650                                                                  | .480    |
| 430              | 7.1 | 1.98x10 <sup>9</sup> | .430            | .640                                                   | .600    | -                                                                     | -       |
| 478              | 7.1 | 1.75x10 <sup>9</sup> | .450            | .560                                                   | .600    | -                                                                     | -       |
| 502              | 7.1 | 1.79x10 <sup>9</sup> | .440            | .600                                                   | .520    | .610                                                                  | .460    |
| 574              | 7.1 | 1.62x10 <sup>9</sup> | .440            | .610                                                   | .530    | -                                                                     | -       |
| 622 <sup>o</sup> | 7.2 | 1.73x10 <sup>9</sup> | .450            | .560                                                   | .500    | .470                                                                  | .460    |
| 670*             | 7.1 | 1.80x10 <sup>9</sup> | .460            | .860                                                   | .800    | .620                                                                  | .480    |
| 742*             | 7.2 | 3.00x10 <sup>9</sup> | .510            | .910                                                   | .900    | -                                                                     | -       |

<sup>o</sup> In this run the air supply varied and especially at 622 h more air than the normal 200-220cc/min was being pumped through.

\* The temperature had increased to 33°C during the night because of circulation pump failure.

<sup>†</sup> A 1:10 dilution of the washed bacterial suspension with the turbidity adjusted to OD 1.040 was used.





Table 10. Growth of *E. coli* in continuous culture. (See Fig. 12)

| Time<br>H | Viable<br>count/ml   | Turbidity<br>OD | Relative dehydrogenase activity                        |         |                                                                       |         |
|-----------|----------------------|-----------------|--------------------------------------------------------|---------|-----------------------------------------------------------------------|---------|
|           |                      |                 | Sample from chemostat<br>Incubation: 20 min at<br>32°C |         | Washed bacteria <sup>†</sup> , OD<br>1.040 Incubation:<br>2 h at 32°C |         |
|           |                      |                 | Glucose +<br>yeast extract                             | Glucose | Glucose +<br>yeast extract                                            | Glucose |
| 0         | 2.9x10 <sup>6</sup>  | 0               | 0                                                      | 0       | -                                                                     | -       |
| 72        | 1.74x10 <sup>9</sup> | .370            | .776                                                   | .630    | .690                                                                  | .550    |
| 108       | -                    | -               | -                                                      | -       | -                                                                     | -       |
| 120       | 1.58x10 <sup>9</sup> | .350            | .700                                                   | .680    | .770                                                                  | .560    |
| 168       | 1.55x10 <sup>9</sup> | .370            | .620                                                   | .610    | .580                                                                  | .430    |
| 192       | 1.64x10 <sup>9</sup> | .360            | .690                                                   | .610    | .670                                                                  | .370    |
| 216       | 1.82x10 <sup>9</sup> | .370            | .620                                                   | .600    | .630                                                                  | .460    |
| 240       | 1.86x10 <sup>9</sup> | .410            | .740                                                   | .740    | .555                                                                  | .480    |
| 288       | Switch               | to complete     | medium                                                 |         |                                                                       |         |
| 312       | 3.90x10 <sup>9</sup> | .640            | .500                                                   | 1.300   | .695                                                                  | .554    |
| 360       | 3.90x10 <sup>9</sup> | .740            | .800                                                   | 1.250   | .795                                                                  | .750    |

<sup>†</sup> A 1:10 dilution of the washed bacterial suspension with the turbidity adjusted to OD 1.040 was used.



Table 11. Growth of *E. coli* in continuous culture. (See Fig. 13)

| Time<br>H | Viable<br>count/ml   | Turbidity<br>OD | Relative dehydrogenase activity                                    |         |         |           |            |
|-----------|----------------------|-----------------|--------------------------------------------------------------------|---------|---------|-----------|------------|
|           |                      |                 | Sample from<br>chemostat<br>Incubation: 20<br>min at 32°C          | Glucose | Glucose | Na malate | Na lactate |
|           |                      |                 | Washed bacteria <sup>†</sup> , OD 1.040<br>Incubation: 2 h at 32°C |         |         |           |            |
| 0         | 4.8x10 <sup>6</sup>  | 0               | 0                                                                  | 0       | -       | -         | -          |
| 18        | -                    | .230            | .390                                                               | .390    | -       | -         | -          |
| 24        | 1.02x10 <sup>9</sup> | .252            | .114                                                               | .397    | -       | -         | -          |
| 42        | 2.10x10 <sup>9</sup> | .380            | 0                                                                  | .520    | -       | -         | -          |
| 66        | -                    | .380            | -                                                                  | -       | .580    | .280      | .420       |
| 90        | 3.10x10 <sup>9</sup> | .386            | 0                                                                  | .590    | .520    | -         | .387       |
| 162       | 3.24x10 <sup>9</sup> | .420            | 0                                                                  | .620    | .550    | .282      | .394       |
| 186       | 2.50x10 <sup>9</sup> | .420            | 0                                                                  | .630    | .500    | .270      | .350       |
| 210       | -                    | .440            | -                                                                  | -       | .516    | .315      | .398       |
| 234       | 2.28x10 <sup>9</sup> | .440            | 0                                                                  | .640    | .460    | .258      | .360       |
| 258       | -                    | .438            | -                                                                  | -       | .545    | .235      | .522       |
| 330       | 1.66x10 <sup>9</sup> | .320            | .113                                                               | .500    | -       | -         | -          |
| 334       | 1.68x10 <sup>9</sup> | .353            | .132                                                               | .540    | -       | -         | -          |
| 358       | 2.10x10 <sup>9</sup> | .432            | 0                                                                  | .640    | .508    | .300      | .400       |

<sup>†</sup> A 1:10 dilution of the washed bacterial suspension with the turbidity adjusted to OD 1.040 was used.



Table 12. Growth of *E. coli* in continuous culture. (See Fig. 14)

| Time<br>H | Viable<br>count/ml   | Turbidity<br>OD | Relative dehydrogenase activity                           |         |                                                                    |           |            |
|-----------|----------------------|-----------------|-----------------------------------------------------------|---------|--------------------------------------------------------------------|-----------|------------|
|           |                      |                 | Sample from<br>chemostat<br>Incubation: 20<br>min at 32°C |         | Washed bacteria <sup>†</sup> , OD 1.040<br>Incubation: 2 h at 32°C |           |            |
|           |                      |                 | Water                                                     | Glucose | Glucose                                                            | Na malate | Na lactate |
| 0         | 6.8x10 <sup>6</sup>  | 0               | 0                                                         | 0       | -                                                                  | -         | -          |
| 24        | 2.03x10 <sup>9</sup> | .360            | 0                                                         | .385    | .390                                                               | .340      | .370       |
| 72        | 2.89x10 <sup>9</sup> | .420            | 0                                                         | .600    | .460                                                               | .250      | .370       |
| 132       | 2.05x10 <sup>9</sup> | .404            | 0                                                         | .596    | .500                                                               | .275      | .400       |
| 192       | 2.98x10 <sup>9</sup> | .420            | 0                                                         | .540    | .476                                                               | .305      | .380       |
| 216       | 2.10x10 <sup>9</sup> | .440            | 0                                                         | .500    | .410                                                               | .260      | .375       |
| 264       | 1.90x10 <sup>9</sup> | .460            | 0                                                         | .560    | .420                                                               | .270      | .320       |
| 312       | 2.00x10 <sup>9</sup> | .460            | 0                                                         | .546    | .450                                                               | .250      | .350       |
| 384       | 2.00x10 <sup>9</sup> | .452            | 0                                                         | .540    | -                                                                  | -         | -          |
| 432       | 2.21x10 <sup>9</sup> | .460            | 0                                                         | .536    | .480                                                               | .320      | .380       |
| 480       | 2.10x10 <sup>9</sup> | .458            | 0                                                         | .540    | -                                                                  | -         | -          |
| 528       | 2.10x10 <sup>9</sup> | .456            | 0                                                         | .536    | -                                                                  | -         | -          |
| 552       | 2.01x10 <sup>9</sup> | .454            | 0                                                         | .530    | -                                                                  | -         | -          |
| 624*      | 2.24x10 <sup>9</sup> | .458            | 0                                                         | .540    | .490                                                               | .330      | .380       |
| 668       | 3.20x10 <sup>9</sup> | .665            | .580                                                      | .880    | -                                                                  | -         | -          |
| 716       | 3.00x10 <sup>9</sup> | .685            | .600                                                      | .930    | -                                                                  | -         | -          |
| 740       | 3.00x10 <sup>9</sup> | .695            | .630                                                      | .980    | .700                                                               | .200      | .730       |

<sup>†</sup> A 1:10 dilution of the washed bacterial suspension with the turbidity adjusted to OD 1.040 was used.

\* Change to complete broth.





Table 13. The effect of incubation time for 60 and 120 min in illustrating dehydrogenase damage of E. coli grown in the chemostat. (See Fig. 15)

|                                     |                            |       |                            |       |                            |       |
|-------------------------------------|----------------------------|-------|----------------------------|-------|----------------------------|-------|
| H in chemostat                      | 334                        |       | 502                        |       | 502                        |       |
| Chlorine ppm                        | 1.5                        |       | 1.5                        |       | 1.0                        |       |
| Incubation min                      | 60                         | 120   | 60                         | 120   | 60                         | 120   |
| <hr/>                               |                            |       |                            |       |                            |       |
| Exposure to chlorine at 32°C (min). |                            |       |                            |       |                            |       |
| <hr/>                               |                            |       |                            |       |                            |       |
| 0                                   | 1.0x10 <sup>9</sup> (100)  |       | 9.4x10 <sup>8</sup> (100)  |       | 9.4x10 <sup>8</sup> (100)  |       |
| 0                                   | .530                       | .650T | .480                       | .610T | .480                       | .610T |
| 0                                   | .460                       | .480G | .410                       | .460G | .410                       | .460G |
| 1/2                                 | .230                       | .270T | .350                       | .440T | .370                       | .470T |
| 3/4                                 | 3.6x10 <sup>8</sup> (36.0) |       | 3.5x10 <sup>8</sup> (37.2) |       | -                          |       |
| 1                                   | .090                       | .190G | .210                       | .250G | .286                       | .300G |
| 1 1/2                               | .210                       | .260T | .326                       | .370T | .370                       | .430T |
| 2                                   | .078                       | .180G | .200                       | .250G | .280                       | .290G |
| 2 1/2                               | .210                       | .260T | .280                       | .330T | .370                       | .410T |
| 3                                   | 3.3x10 <sup>8</sup> (33.0) |       | .180                       | .234G | .260                       | .270G |
| 3 1/2                               | .060                       | .170G | .260                       | .300T | .350                       | .390T |
| 4                                   | .200                       | .260T | .160                       | .230G | .260                       | .270G |
| 4 1/2                               | .050                       | .160G | .260                       | .300T | .350                       | .390T |
| 5                                   | .200                       | .260T | 1.8x10 <sup>8</sup> (19.1) |       | 5.5x10 <sup>8</sup> (58.4) |       |
| 5 1/2                               | 2.7x10 <sup>8</sup> (27.0) |       |                            |       |                            |       |

In the table (T) indicates glucose + yeast extract, (G) indicates glucose substrate. % survivors given in brackets.



Table 14. Metabolic damage and death of *E. coli* grown in the chemostat on exposure to 1.5 and 2.0 ppm chlorine. (See Fig. 16).

| H in chemostat                     | 622                      |                          | 670                      |                         |
|------------------------------------|--------------------------|--------------------------|--------------------------|-------------------------|
| Chlorine ppm                       | 1.5                      | 2.0                      | 1.5                      | 2.0                     |
| Incubation min                     | 120                      |                          | 120                      |                         |
| <hr/>                              |                          |                          |                          |                         |
| Exposure to chlorine at 32°C (min) |                          |                          |                          |                         |
| <hr/>                              |                          |                          |                          |                         |
| 0                                  | $1.1 \times 10^9$ (100)  | $1.2 \times 10^9$ (100)  | $1.2 \times 10^9$ (100)  |                         |
| 0                                  | .470                     | .470T                    | .620                     | .620T                   |
| 0                                  | .460                     | .460G                    | .480                     | .480G                   |
| 1/2                                | .290                     | .090T                    | .180                     | .010G                   |
| 3/4                                | $2.6 \times 10^8$ (26.0) | $7.9 \times 10^7$ (7.9)  | .250                     | .110T                   |
| 1                                  | .204                     | .010G                    | $2.4 \times 10^8$ (20.0) | $7.2 \times 10^7$ (6.0) |
| 1 1/2                              | .270                     | .050T                    | .150                     | OG                      |
| 2                                  | .190                     | OG                       | .230                     | .050T                   |
| 2 1/2                              | .250                     | .010T                    | .140                     | OG                      |
| 3                                  | $2.3 \times 10^8$ (23.0) | $1.8 \times 10^7$ (1.8)  | .210                     | .030T                   |
| 3 1/2                              | .170                     | OG                       | $2.8 \times 10^8$ (23.4) | $2.9 \times 10^7$ (2.4) |
| 4                                  | .210                     | OT                       | .120G                    | OG                      |
| 4 1/2                              | .140                     | OG                       | .190                     | .020T                   |
| 5                                  | .200                     | OT                       | $2.0 \times 10^8$ (16.7) | $1.0 \times 10^7$ (0.8) |
| 5 1/2                              | $1.8 \times 10^8$ (18.0) | $1.15 \times 10^6$ (0.1) |                          |                         |

In the table (T) indicates glucose + yeast extract, (G) indicates glucose substrate. % survivors in brackets.





Table 15. Further evidence of metabolic damage and death of E. coli on exposure to chlorine at 32°C. Bacteria grown in the chemostat. (See Fig. 11).

| H in chemostat                     | 310                      | 502                     | 574                      | 622                      |
|------------------------------------|--------------------------|-------------------------|--------------------------|--------------------------|
| Chlorine ppm                       | 1.4                      | 2.0                     | 1.5                      | 2.0                      |
| Incubation min                     | 60                       | 60                      | 120                      | 90                       |
|                                    |                          |                         | 120                      | 90                       |
|                                    |                          |                         | 90                       | 120                      |
| Exposure to chlorine at 32°C (min) |                          |                         |                          |                          |
| 0                                  | $9.0 \times 10^8$ (100)  | $9.0 \times 10^8$ (100) | $9.2 \times 10^8$ (100)  | $1.0 \times 10^9$ (100)  |
| 0                                  | .400T                    | .480T                   | .560                     | .428                     |
| 0                                  | .360G                    | .410                    | .460G                    | .390                     |
| 1/2                                | .190T                    | .120                    | .380                     | .250                     |
| 3/4                                | $2.7 \times 10^8$ (30.0) | -                       | $4.6 \times 10^8$ (50.0) | $2.6 \times 10^8$ (25.0) |
| 1                                  | .070G                    | .010                    | .248                     | .170                     |
| 1 1/2                              | .180T                    | -                       | .348                     | .240                     |
| 2                                  | .046G                    | $8.6 \times 10^6$ (0.9) | .250                     | .150                     |
| 2 1/2                              | .160T                    | -                       | .308                     | .210                     |
| 3                                  | $1.6 \times 10^8$ (17.8) | -                       | $3.5 \times 10^7$ (3.8)  | $2.3 \times 10^8$ (23.0) |
| 3 1/2                              | .020G                    | -                       | .250                     | .130                     |
| 4                                  | .160T                    | -                       | .308                     | .190                     |
| 4 1/2                              | 0                        | -                       | .226                     | .106                     |
| 5                                  | .160T                    | -                       | .300                     | .170                     |
| 5 1/2                              | -                        | -                       | $3.3 \times 10^7$ (3.6)  | $1.8 \times 10^8$ (18.0) |

In the table (T) indicates glucose + yeast extract, (G) indicates glucose substrate. % survivors in brackets.



Table 16. Metabolic damage of *E. coli* with varying growth histories on exposure to 1 ppm chlorine (Note that dehydrogenase activity indicated by all substrates decreases, except with cultures grown in complete broth batch culture. An increase of activity is apparent when glucose only is used as the substrate). See Fig. 18.

| Exposure to Culture history, optical density with a no. 42 filter and substrates |                           |      |   |      |      |                                 |      |    |      |      |      |      |      |
|----------------------------------------------------------------------------------|---------------------------|------|---|------|------|---------------------------------|------|----|------|------|------|------|------|
| 1 ppm                                                                            | Chemostat - Minimal broth |      |   |      |      | Batch cultures - Complete broth |      |    |      |      |      |      |      |
| chlorine at 32°C (min)                                                           | 1                         |      | 2 |      |      | 3                               |      |    | 4    |      | 5    |      |      |
| 0                                                                                | W                         | 0    | - | *    |      | W                               | +    |    | W    | +    | W    | +    |      |
| 0                                                                                | B                         | .692 | B | .650 | .550 | B                               | .620 | -  | B    | .728 | B    | .692 |      |
| 0                                                                                | YC                        | .328 | Y | .550 | .510 | -                               |      | YC | .328 | -    | YC   | .332 |      |
| 0                                                                                | G                         | .080 | G | .520 | .500 | -                               |      | G  | .088 | G    | .100 | G    | .088 |
| 1/4                                                                              | G                         | .020 | Y | .540 | .530 | -                               |      | -  |      | G    | .180 | G    | .132 |
| 1/2                                                                              | B                         | .184 | B | .470 | .470 | B                               | .328 | G  | .168 | B    | .488 | B    | .352 |
| 3/4                                                                              | YC                        | .104 | G | .360 | .360 | -                               |      | -  |      | G    | .180 | G    | .128 |
| 1                                                                                | G                         | .020 | Y | .338 | .338 | B                               | .320 | YC | .160 | B    | .436 | YC   | .236 |
| 1 1/4                                                                            | -                         |      | B | .308 | .308 | -                               |      | -  |      | -    |      | -    |      |
| 1 1/2                                                                            | B                         | .148 | G | .222 | .200 | -                               |      | G  | .188 | -    |      | B    | .340 |
| 1 3/4                                                                            | -                         |      | Y | .242 | .230 | -                               |      | -  |      | -    |      | -    |      |
| 2                                                                                | G                         | 0    | B | .232 | .228 | B                               | .288 | YC | .156 | B    | .408 | G    | .136 |
| 2 1/2                                                                            | YC                        | .076 | G | .100 | .020 | -                               |      | G  | .196 | G    | .180 | YC   | .256 |
| 3                                                                                | B                         | .128 | - |      |      | B                               | .288 | YC | .148 | B    | .392 | G    | .136 |
| 3 1/2                                                                            | -                         |      | - |      |      | -                               |      | G  | .200 | G    | .180 | B    | .324 |
| 4                                                                                | -                         |      | - |      |      | B                               | .280 | YC | .148 | -    |      | -    |      |
| 4 1/2                                                                            | -                         |      | - |      |      | -                               |      | -  |      | -    |      | -    |      |
| 5                                                                                | -                         |      | - |      |      | B                               | .280 | -  |      | -    |      | -    |      |

\* Readings obtained after one hour incubation

(Complex substrates were used for the tetrazolium test as follows: W - water, G - glucose; Y - yeast extract; C - casein digest; B = G + Y + C, which is complete broth.)





Table 17. Metabolic damage and death of *E. coli* grown in the chemostat on exposure to 1.5 and 2.0 ppm chlorine. (See Fig. 12 and 17a, b and c.)

| H in chemostat               | 72         | 120               | 168               | 192               |
|------------------------------|------------|-------------------|-------------------|-------------------|
| Chlorine ppm                 | 2.0        | 1.5               | 2.0               | 1.5               |
| Exposure to chlorine at 32°C |            |                   |                   |                   |
| min                          | Substrates |                   |                   |                   |
| 0                            | Count      | $1.0 \times 10^9$ | $1.1 \times 10^9$ | $9.0 \times 10^8$ |
| 0                            | T          | .690              | .580              | .670              |
| 0                            | G          | .550              | .430              | .370              |
| 1/2                          | G          | .010              | .020              | .020              |
| 3/4                          | T          | .018              | .028              | .100              |
| 1                            | Count      | $3.3 \times 10^6$ | $4.1 \times 10^7$ | $8.1 \times 10^7$ |
|                              |            | (.3)              | (4.1)             | (7.4)             |
|                              |            | $1.4 \times 10^8$ | $1.5 \times 10^8$ | $5.8 \times 10^8$ |
|                              |            | (14.0)            | (15.0)            | (52.7)            |
| 1 1/2                        | G          | .010              | 0                 | 0                 |
| 2                            | T          | .012              | .010              | .050              |
| 2 1/2                        | G          | 0                 | 0                 | 0                 |
| 3                            | T          | .012              | 0                 | 0                 |
| 3 1/2                        | Count      | $6.9 \times 10^5$ | $9.2 \times 10^6$ | $2.8 \times 10^7$ |
|                              |            | (.07)             | (0.9)             | (2.5)             |
|                              |            | $1.3 \times 10^8$ | $1.5 \times 10^8$ | $5.5 \times 10^8$ |
|                              |            | (13.0)            | (15.0)            | (50.0)            |
| 4                            | G          | 0                 | 0                 | 0                 |
| 4 1/2                        | T          | 0                 | 0                 | 0                 |
| 5                            | Count      | $3.5 \times 10^5$ | $2.6 \times 10^6$ | $2.7 \times 10^7$ |
|                              |            | (.04)             | (0.3)             | (2.5)             |
|                              |            | $1.1 \times 10^8$ | $1.4 \times 10^8$ | $3.8 \times 10^8$ |
|                              |            | (11.0)            | (14.0)            | (34.5)            |
|                              |            |                   |                   | $1.6 \times 10^8$ |
|                              |            |                   |                   | (17.8)            |
|                              |            |                   |                   | $2.0 \times 10^8$ |
|                              |            |                   |                   | (22.2)            |

In the table (T) indicates glucose + yeast extract, (G) indicates glucose substrate.  
% survivors in brackets.





Table 17 (cont.) Metabolic damage and death of *E. coli* grown in the chemostat on exposure to 1.5 and 2.0 ppm chlorine. (See Figs. 12 and 17a, b and c.)

| H in chemostat               | 216        | 240                 | 312                 | 360                  |
|------------------------------|------------|---------------------|---------------------|----------------------|
| Chlorine ppm                 | 2.0        | 1.5                 | 2.0                 | 1.5                  |
| Exposure to chlorine at 32°C |            |                     |                     |                      |
| min                          | Substrates |                     |                     |                      |
| 0                            | Count      | 1.0x10 <sup>9</sup> | 8.5x10 <sup>9</sup> | 1.1x10 <sup>9</sup>  |
| 0                            | T          | .630                | .695                | .795                 |
| 0                            | G          | .460                | .554                | .750                 |
| 1/2                          | G          | .020                | .160                | .310                 |
| 3/4                          | T          | .140                | .250                | .380                 |
| 1                            | Count      | 9.7x10 <sup>7</sup> | 1.3x10 <sup>8</sup> | 1.46x10 <sup>8</sup> |
|                              |            | (9.7)               | (13)                | (17.4)               |
| 1 1/2                        | G          | .020                | .140                | .224                 |
| 2                            | T          | .120                | .230                | .300                 |
| 2 1/2                        | G          | .015                | .120                | .190                 |
| 3                            | T          | .070                | .090                | .200                 |
| 3 1/2                        | Count      | 4.0x10 <sup>7</sup> | 6.8x10 <sup>7</sup> | 5.3x10 <sup>5</sup>  |
|                              |            | (4.0)               | (8.6)               | (0.06)               |
| 4                            | G          | 0                   | .080                | 0                    |
| 4 1/2                        | T          | .024                | .180                | .120                 |
| 5                            | Count      | 1.9x10 <sup>7</sup> | 6.7x10 <sup>7</sup> | 1.9x10 <sup>7</sup>  |
|                              |            | (1.9)               | (6.7)               | (2.2)                |
|                              |            |                     | (0.05)              | (1.4)                |
|                              |            |                     |                     | (5.4)                |

In the table (T) indicates glucose + yeast extract, (G) indicates glucose substrate. % survivors given in brackets. At 312 and 360 complete broth was being fed into the culture vessel.



Table 22. The involvement of glucose, malic and lactic dehydrogenase in death of E. coli by chloramine-T. E. coli grown in the chemostat in minimal medium. (See Fig. 14 and 11).

| H in chemostat                       | 192                      | 216                      | 216               | 216               |
|--------------------------------------|--------------------------|--------------------------|-------------------|-------------------|
| Chlorine ppm                         | 10                       | 20                       | 30                | 50                |
| Exposure to chloramine-T at 20°C min |                          |                          |                   |                   |
| 0 Count                              | $1.4 \times 10^9$ (100)  | $1.0 \times 10^9$ (100)  | $1.0 \times 10^9$ | $1.0 \times 10^9$ |
| 0 Water                              | 0                        | 0                        | -                 | -                 |
| 0 G                                  | .476                     | .410                     | .410              | .410              |
| 0 M                                  | .305                     | .260                     | .260              | .260              |
| 0 L                                  | .380                     | .375                     | .375              | .375              |
| 1/2 G                                | .460                     | .400                     | .400              | .320              |
| 3/4 Count                            | $1.3 \times 10^9$ (92.9) | $1.0 \times 10^9$ (100)  | -                 | -                 |
| 1 M                                  | .300                     | .240                     | .240              | .080              |
| 1 1/2 L                              | .380                     | .310                     | .280              | .050              |
| 1 3/4 Count                          | $1.4 \times 10^9$ (100)  | $7.6 \times 10^8$ (76.0) | -                 | -                 |
| 2 G                                  | .460                     | .340                     | .310              | .010              |
| 2 1/2 M                              | .280                     | .190                     | .140              | 0                 |
| 2 3/4 Count                          | $1.3 \times 10^9$ (92.9) | $3.2 \times 10^8$ (32.0) | -                 | -                 |
| 3 L                                  | .360                     | .200                     | .180              | 0                 |
| 3 1/2 G                              | .440                     | .180                     | .100              | 0                 |
| 4 M                                  | .260                     | .070                     | 0                 | 0                 |
| 4 1/2 L                              | .350                     | .130                     | .064              | 0                 |
| 5 Count                              | $1.1 \times 10^9$ (78.6) | $1.0 \times 10^8$ (10.0) | -                 | -                 |

In the table (G) indicates glucose, (M) indicates sodium malate, and (L) indicates sodium lactate substrate. % survivors given in brackets.





Table 23. Evidence that glucose may protect E. coli grown in minimal broth batch culture for 24 h at 32°C, from being killed by 2.0 ppm chlorine at 20°C and pH 7.2. (Where the E. coli suspension was preincubated with 1 ml of 1M glucose/10 ml of washed bacteria, the chlorine was contained in 89 ml buffer, 11 ml of glucose-bacterial mixture were inoculated. Since each 10 ml amount of bacteria withdrawn from the disinfectant suspension would carry 1/10 ml of the glucose added, this was compensated for in preparing substrate by adding only 0.4 ml glucose and 0.1 ml buffer. In the case of sodium malate and sodium lactate compensation was not possible).

| Exposure to<br>2.0 ppm<br>chlorine at<br>20°C min | Substrate 1/24M | No<br>preincubation<br>with glucose | Preincubate <u>E. coli</u><br>for 5 min at 20°C<br>with 1 ml of 1M<br>glucose/10 ml. |
|---------------------------------------------------|-----------------|-------------------------------------|--------------------------------------------------------------------------------------|
| 0                                                 | Water           | 0                                   | -                                                                                    |
| 0                                                 | Glucose         | .194                                | .194                                                                                 |
| 0                                                 | Na malate       | .260                                | .270                                                                                 |
| 0                                                 | Na lactate      | .480                                | .110                                                                                 |
| 0                                                 | Count and %     | $1.0 \times 10^9$ (100)             | $1.0 \times 10^9$ (100)                                                              |
| 1/2                                               | Glucose         | .080                                | .200                                                                                 |
| 3/4                                               | Na malate       | .020                                | .194                                                                                 |
| 1                                                 | Count and %     | $3.0 \times 10^8$ (30.0)            | $5.2 \times 10^8$ (52.0)                                                             |
| 1 1/4                                             | Na lactate      | .130                                | .230                                                                                 |
| 1 1/2                                             | Glucose         | .050                                | .194                                                                                 |
| 1 3/4                                             | Na malate       | .020                                | .180                                                                                 |
| 2                                                 | Count and %     | $2.9 \times 10^8$ (29.0)            | $3.7 \times 10^8$ (37.0)                                                             |
| 2 1/4                                             | Na lactate      | .080                                | .220                                                                                 |
| 2 1/2                                             | Glucose         | .030                                | .200                                                                                 |
| 2 3/4                                             | Na malate       | 0                                   | .180                                                                                 |
| 3 1/4                                             | Na lactate      | .050                                | .220                                                                                 |
| 4                                                 | Count and %     | $1.7 \times 10^8$ (17.0)            | $2.2 \times 10^8$ (22.0)                                                             |



Table 25. Survival of E. coli on exposure to 2.0 ppm chlorine at 32°C and pH 7.2. E. coli grown in the chemostat while operating on minimal broth. (See Fig. 14 and 21).

| H in chemostat                                    | 24                      | 72                      | 384                     | 432                     |
|---------------------------------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| Exposure to<br>2.0 ppm<br>chlorine at 20°C<br>min |                         |                         |                         |                         |
| 0                                                 | $1.4 \times 10^9$ (100) | $1.3 \times 10^9$ (100) | $1.3 \times 10^9$ (100) | $1.4 \times 10^9$ (100) |
| 3/4                                               | $6.8 \times 10^7$ (4.9) | $1.1 \times 10^7$ (0.8) | $4.7 \times 10^7$ (3.6) | $1.1 \times 10^7$ (0.8) |
| 1 3/4                                             | $3.7 \times 10^7$ (2.6) | $6.4 \times 10^6$ (0.5) | $4.1 \times 10^7$ (3.2) | $8.3 \times 10^6$ (0.6) |
| 2 3/4                                             | $5.7 \times 10^6$ (0.4) | $2.2 \times 10^6$ (0.2) | $3.8 \times 10^7$ (2.9) | $5.1 \times 10^6$ (0.4) |
| 5                                                 | $5.4 \times 10^6$ (0.4) | $2.1 \times 10^6$ (0.2) | $1.8 \times 10^7$ (1.4) | $5.0 \times 10^6$ (0.4) |







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